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OFFICE OF NAVAL RESEARCH

Contract N00014-77-C-0340

Task No. NR 206-003

ANNUAL REPORT NO. 2

Chemical and Biochemical Studies of PGB_x

by

Thomas M. Devlin, Ph.D.

Department of Biological Chemistry
Hahnemann Medical College & Hospital
Philadelphia, Pa. 19102

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Department of Biological Chemistry

Hahnemann Medical College and Hospital

I. Introduction:

In September 1979 two existing contracts, one to the Department of Biological Chemistry, Hahnemann Medical College, and the other contract for studies conducted at the Naval Air Development Center (NADC), Warminster, Pa., were combined into one contract awarded to the Hahnemann Medical College and Hospital. The combined contract led to a more integrated research activity and an increase in professional staff interaction during 1980 even though the two groups are physically separated and each had separate goals and objectives. The specific activities of the two groups can be clearly delineated and are presented separately in this report.

In addition to the activities supported by this contract at the NADC are the separately funded activities at the NADC directed by Dr. Herman Shmukler. The NADC groups function as an integrated total unit rather than as two separate activities. On this basis, the report submitted here for the NADC activities overlaps the report submitted by Dr. Shmukler because it was deemed important to prepare a comprehensive report for the activity at NADC.

Both the Hahnemann and NADC units perform a service function. It is the responsibility of the Hahnemann group to carry out assays of samples submitted by other contractors who are conducting purification studies of PGB_x or are preparing analogues of PGB_x. The NADC group is the principal laboratory for preparation of PGB_x serving the contract to the Department of Chemistry, St. Joseph's University, by purifying intermediates in the synthesis of PGB_x as well as preparing PGB_x for

use by other investigators.

II. Report of the Research Program at Hahnemann:

A. Background:

Previous progress reports described the earlier studies from this laboratory confirming the observations of Polis, Polis and Kwong (Proc. Natl. Aca. Sci. USA (1979) 76 1958) that a polomeric form of 15-keto prostaglandin B_1, designated PGB_x, was capable of protecting oxidative phosphorylation of isolated rat liver mitochondria which had been aged at 0° for several days. The complexity of the energy transduction mechanism has been discussed in previous reports as well as the sensitivity of this system to a variety of treatments, chemical agents and perturbations of the membrane. A variety of events could occur during aging periods in vitro any of which could be prevented or reversed by PGB_x. Based on the studies conducted during the last two years it is now considered that the principal effect of PGB_x is on intermediate in the energy transduction mechanism but the possibility of a direct effect on the membrane thus protecting the ATP synthetic activity indirectly has not been ruled out.

The ability of PGB_x to protect oxidative phosphorylation is a very unusual property in that no other compound has been found to have a similar activity particularly at the low concentrations of PGB_x that are effective; some prostaglandins do have nominal activity but none at the low concentration observed with PGB_x. Thus an understanding of the action of PGB_x should have a major impact not only on our understanding of the mechanism of oxidative phosphorylation but also on possible therapeutic approaches to protecting mitochondria from loss of integrity in pathological

conditions where loss of mitochondrial function occurs.

A major effort was undertaken in 1980 by several laboratories to purify PGB_x and analogues of PGB_x. Samples of purified PGB_x, various analogue derivatives and a number of other compounds have been tested to determine if they have an activity similar to PGB_x. As reported previously PGB_x has a biphasic effect on mitochondria protecting oxidative phosphorylation at low concentrations but causing an inhibition at higher concentrations. A number of samples have been tested which have lower inhibitory activity but also lower activity in protecting oxidative phosphorylation from loss. It is still not known whether the protective and inhibitory activities are the function of one or two different compounds in the sample.

In 1978, we demonstrated (Ohnishi, S.T. and Devlin, T.M., Biochem. Biophys. Res. Commun. (1979) 89 240) that PGB_x had ionophoretic activity, capable of translocating Ca^{++} across biological membranes and partitioning Ca^{++} between an aqueous and an organic phase. Studies of the ionophoretic properties have continued with the evidence suggesting that PGB_x serves as an ionophore and not to form channels in the membrane. The ionophoretic properties of PGB_x have been confirmed by Weissmann, et al (Weissmann, G., Anderson, P., Serhan, C., Samuelson, E., and Goodman, E. (1980) Proc. Natl. Acad. Sci. USA 77 1506). The relationship between the ionophoretic activity and the protective action on oxidative phosphorylation of PGB_x has not been delineated. Extensive studies have been conducted in order to demonstrate a possible relationship or lack of relationship but the results

are inconclusive. In addition it is not certain whether the two activities are due to the same or different compounds in samples of PGB_x.

B. Progress Report:

This report covers the period September 1, 1979 through August 30, 1980. For those phases of the investigation which have been completed experimental procedures and results are presented but where work is still in progress and results are preliminary the details are presented in summary form only.

(1) Summary: Over one hundred different samples were tested for their ability to protect isolated rat liver mitochondria from the loss of oxidative phosphorylation; samples were tested at several concentrations and with several different mitochondrial preparations. Results were reported to individuals submitting samples. Several modifications of the assay system have permitted a quantitative comparison between samples tested and a stock sample of PGB_x. There is a direct relationship between mitochondrial protein and quantity of PGB_x in the assay system. Test results were reported on the basis of the ratio of amount of sample to produce 50% protection and mitochondrial protein. The assay system has been modified to permit the use of fresh mitochondria which are aged by preincubation in the absence of ATP but in the presence of phosphate. This has reduced some of the erratic results of the previous assay system. Oligomers of the ethyl-analogue (from Dr. G. Nelson) have been evaluated; the most active fraction is approximately 40% as active as the PGB_x but does have less inhibitory activity.

PGB_x apparently binds specifically to the proteolipid of the F_0 portion of the F_1-F_0 ATPase of mitochondria. This was demonstrated by competitive binding studies with dicylohexyl-carbodiimide (DCCD). The F_0 complex is involved in proton movements across the mitochondrial membrane and interaction of PGB_x on the proteolipid portion would explain the observed inhibition of respiration with freshly isolated mitochondria. This proposal for a site of action does not, however, explain the inhibition of uncoupler stimulated respiration.

A comprehensive study of the binding of Ca^{++} by PGB_x and serum albumin by PGB_x has been completed. The results demonstrate that PGB_x binds high affinity sites on serum albumin in an area identical to or overlaping Ca^{++} binding sites. Physical chemical studies on the binding of Ca^{++} by PGB_x in an aqueous system indicates that a similar interaction occurs in the ionophoretic activity of PGB_x in the lipid membrane.

The ionophoretic activity of PGB_x with isolated liposomes has been evluated. The pH of optima activity is approximately 6.9 but in contrast to other ionophores there seems to be a maximum pH with decreasing activity at both higher and lower pHs. The temperature profile supports the proposal that PGB_x is a true ionophore and not a channel former in the membrane. The ionophoretic activity of fractions of PGB_x and of the ethyl-analogue demonstrate that the activity is a function of the molecular weight; the higher molecular weight fractions have higher ionophoretic activity. The ethyl analogue has nearly the same ionophoretic activity as PGB_x.

In very preliminary studies with an isolated perfused heart system, PGB_x apparently protects the heart during periods of anoxia permitting the heart to resume its function when O_2'' is administered.

(2) <u>Assay of Samples:</u> During the past year over one hundred samples have been tested for their ability to protect isolated mitochondria from the loss of oxidative phosphorylation. Samples were usually tested with two to four different mitochondrial preparations and at three to five different concentrations. Samples were received from and reports of results were submitted to Dr. H. Shmukler (NADC), Dr. G. Nelson (St. Joseph's University), Dr. R. Doskotch (Ohio State University), Dr. K. Biemann (MIT) and Dr. D. Trainor (Columbia University). This report does not contain detailed results on all the samples; any activity of the samples tested should be reported in the Progess Reports from the submitting Institutions.

During this past year a major modification of the assay system has been carried out. The original studies of Polis as well as those in this laboratory indicated the requirement to age isolated rat liver mitochondria in their isolation medium at 0° for several days before assay. This aging period apparently labelized some component of the energy transduction mechanism such that when the mitochondria were preincubated for short periods (5 to 10 minutes) at 30° there would be a loss of phosphorylating capacity. The loss could be prevented by the presence of PGB_x during the preincubation. The need to age for several days presented problems in that there was a great variability in the results

and thus one could not be certain that a particular mitochondrial preparation could be used for an assay. An evaluation of the effect of preincubating rat liver mitochondria at 30° under varying conditions led to the conclusion that it was possible to use freshly isolated mitochondria if the mitochondria were preincubated in the presence of inorganic phosphate and absence of ATP. A system has been developed in which freshly isolated mitochondria can be preincubated in the absence or presence of PGB_x to demonstrate the protective effect. Results with this system are more reproducible than with two to five day aged mitochondria but we still encounter some lack of consistency. The system is presented in Figure 1 which demonstrates the effect of increasing concentrations of PGB_x on phosphate uptake. In the experiment presented in Figure 1, the mitochondria after preincubating for 45 minutes in the presence of ATP were able to catalyze the uptake of 33μ moles of phophate in a 30 minute incubation period. In the absence of ATP, however, there was a significant loss of the phosphorylating capacity as indicated. PGB_x was able to protect the mitochondria from this loss. The system permits a determination of the percent protection as indicated on the right hand ordinate and a method to compare the protective effect of other samples. The protection by ATP is presumably due to its ability to maintain the integrity of the phosphorylating mechanism and to maintain the mitochondrial respiratory chain in a reduced state. The absence of ATP or any addition which leads to a depletion of adeninenucleotides of the mitochondria causes a loss of phosphorylating activity. It should be noted that this assay system does not require the

presence of factors such as NAD, cytochrome c or bovine serum albumin (BSA) which are frequently added to improve the activity of oxidative phosphorylation.

As discussed below in more detail, the effect of PGB_x was related directly to the ratio of PGB_x and mitochondrial protein rather than to the concentration of PGB_x. Variations in mitochondrial protein will lead to differences in the degree of protection by the same concnetration of PGB_x. Thus in reporting results on the assay of samples, the concentration of material required to protect 50% based on mg mitochondrial protein was used. For PGB_x to protect mitochondria 50% the value was between 1.7 and 2.4 $\mu g/mg$ mitochondrial protein. PGB_x is usually tested at 20 to 30 $\mu g/2.8$ ml of incubating system.

The assay system also permitted the evaluation of the inhibitory activity of a sample. As presented in Figure 1 at higher concentrations of PGB_x there was a decrease in the protective effect. In fact, if the concentration of PGB_x was raised above 60 $\mu g/2.8$ ml there was a greater loss of activity than in the control. Thus samples can be compared for both their protective and inhibitory activity. An alternative assay for the inhibitory activity is to use freshly prepared mitochondria as shown in Figure 2. There was a close correlation between the inhibitory activity of PGB_x where PGB_x was preincubated in the presence of ATP or the absence of ATP. Without the preincubation there appeared to be somewhat less inhibitory activity. There is no ready explanation for the lack of effect at low PGB_x concentrations either with or without the preincubation in the presence of ATP. It is possible that we are dealing with a single phenomena with a threshold concentra-

tion required beyond which the effect of the PGB_x becomes deliterious but below which it has a positive protective effect or no effect as observed with mitochondria which are not preincubated. A number of samples have been tested in this fashion and some have been found to have reduced levels of inhibition in comparison to PGB_x but they also have reduced protective effects. At this time no fraction of PGB_x has been found to be totally free of inhibitor even though some of the fractions appear to have a lower inhibitor activity than others.

Dr. G. Nelson (St. Joseph's) has prepared an ethyl-analogue of PGB which has been oligomerized and partially purified. The results of the assay of these samples is presented in Table I. It will be noted that none of the samples of the ethyl-analogue were as active as PGB_x. Of particular interest is that the samples have very low inhibitory activity. The samples have also been evaluated for their ionophoretic activity and it will be noted that one fraction of the oligomer is more active than PGB_x.

(3) <u>Studies with Intact Mitochondria and Sub-mitochondrial Particles:</u> Studies of the effect of PGB_x on specific aspects of the energy transduction mechanism of mitochondria have continued. It has been observed that the inhibition of oxidative phosphorylation by PGB_x was dependent on the concentration of mitochondrial protein. This is reported in Figure 3 where the effect of PGB_x on inhibition of phosphorylation is measured at three different levels of mitochondrial protein; with increasing mitochondrial protein there was a decreasing effect of PGB_x. A similar observation has been made with respect to the protec-

tive effect of PGB_x on mitochondrial phosphorylation. Thus we have had to relate the effect of PGB_x and other compounds to the amount of mitochondrial protein rather than to the absolute concentration of PGB_x in the incubation system. The results indicate a direct interaction of PGB_x with a component of the mitochondria with a high affinity constant.

We have found that PGB_x is also tightly bound by serum albumin. As presented in Figure 4 inhibition of phosphorylation by PGB_x can be prevented by increasing concentrations of serum albumin. At a molar ratio of 1 there was no effect of PGB_x on phosphorylation; at ratios lower than 1, PGB_x was inhibitory. This suggests a very specific binding by serum albumin of PGB_x and is reminiscent of the high affinity binding sites for 1   ds on albumin. Further studies on the binding of albumin and PGB_x are described below. This binding by BSA partially explains some of the erratic results observed earlier where BSA could prevent the protective effect of PGB_x. Data in the NADC Report below confirms this proposal.

Studies have also continued on the specific site of action on PGB_x on the energy transduction mechanism. Results reported in the 1979 progress report indicated the possibility of a direct interaction with one of the proteins of the F_1-F_0ATPase. Studies of the effect of PGB_x on the rate of respiration in the presence and absence of ADP or Ca^{++} and on reverse electron transport indicated a site of action on the ATPase. The F_1-F_0ATPase is the enzyme involved in proton translocation across the mitochondrial membrane and reversal of the ATPase activity is considered to be

the mechanism of ATP synthesis. PGB_x inhibited the ATPase activity of sub-mitochondrial particles. Isolation of the F_1 portion, which catalyzes the actual hydrolysis of ATP, however, demonstrated that the PGB_x did not inhibit this aspect of the enzyme. The F_0 portion of the enzyme is considered to be the channel through which protons move from the cytoplasmic side of the membrane into the matrix and driving ATP synthesis. Results with the purified F_1ATPase supported the proposal that PGB_x was specifically binding the F_0 complex. This was demonstrated with sub-mitochondrial particles prepared in the presence of urea which removes the F_1 portion of the complex leaving particles containing the F_0 portion. These particles are essentially uncoupled in that protons can move through the membrane via the F_0 complex. The addition of PGB_x to these particles reinstituted respiratory control, an expected observation if PGB_x is reacting with the F_0. Proof for the interaction of PGB_x with the F_0 complex came from the isolation of the complex and the demonstration that PGB_x apparently binds with one of the proteins. The F_0 complex contains a number of proteins, one a small molecular weight proteolipid which specifically binds DCCD. PGB_x apparently competes with DCCD for binding to the proteolipid of F_0 as shown in Figure 5. These results confirm the original proposal of a site of interaction at the energy transduction mechanism. Since it is considered that proteolipid is involved in the proton movements, PGB_x might be inhibiting a leak of protons across the membrane at low concentrations but at high concentration inhibiting all proton translocations thereby inhibiting ATP synthesis.

This explanation, however, does not explain all the observations. PGB_x also effects the uncoupling due to 2,4-dinitrophenol as reported by Polis et al (op. cit.). Dinitrophenol has been considered to be a protophore, short circuiting the F_1-F_0ATPase. If this is the case, it would be necessary to propose a site of action of dinitrophenol somewhere close to that of the interaction of PGB_x.

The effect of PGB_x on mitochondrial swelling suggests that the compound prevents various types of induced swelling particularly that caused by phosphate. The fine details of this system have not been worked out and it appears that there is a specific cation and/or anion requirement in order to demonstrate the protection. Further studies in this area are currently underway.

(4) <u>Interaction of PGB_x with Calcium and Bovine Serum Albumin:</u>

As indicated above, it was observed that the addition of bovine serum albumin could prevent the protective effect of PGB_x on aged mitochondria or prevent the uncoupling by PGB_x presumably due to binding. Detailed evaluation of this interaction was undertaken as well as the interaction of Ca^{++} with PGB_x as part of a continuing study on the ionophoretic activity of the material. The results of binding as measured by the quenching of flourescence of bovine serum albumin indicated that BSA binds at least two molecules of PGB_x with an affinity constant of 2×10^7/M. This is higher than the affinity of serum albumin for sodium dodecylsulfate. There are apparently two different types of binding--a low affinity and a higher affinity. Calculations of the molar ratio suggest that two molecules of PGB_x of approximate molecular

weight of 2000 are bound per serum albumin, one to the high affinity and one to the low affinity binding site. Similar data was calculated in the presence of liposomes but the results suggest a higher binding ratio of PGB_x to BSA. This could be accounted for by the interaction of PGB_x in the liposomes permitting only a portion of the molecule to interact with the BSA. Thus more than one molecule of PGB_x in the liposome system would be able to interact in contrast to the aqueous system where only two molecules are able to interact. There are known to be ten hydrophobic binding sites on serum albumin and thus it may be able to bind a large number of PGB_x molecules dissolved in the lipid bilayer.

The interaction of serum albumin and PGB_x is prevented by prior treatment of the serum albumin with Ca^{++}. The results of binding experiments suggest that Ca^{++} can prevent the PGB_x binding and PGB_x can prevent Ca^{++} binding. It appears as if both the Ca^{++} and PGB_x binding sites are located close together and presumably in an area close to the chromophore of BSA fluorescence. The results suggest that the binding of PGB_x to BSA may involve not only hydrophobic binding but also ionic charged sites and that the interaction between the three molecules, PGB_x, Ca^{++} and BSA occurs on a first come first occupied basis.

A study of PGB_x interaction with Ca^{++} in an aqueous system has been completed since PGB_x is water soluble and thus has the advantage over other ionophores which are usually only soluble in lipid solvents. The water solubility of PGB_x permitted the measurement of the titration of PGB_x by Ca^{++} in the presence of

a metalochromic indicator, titration of changes in intrinsic fluorescence of PGB_x in addition of Ca^{++} and measurement of Ca^{++} influx across the liposome membrane. Evidence has developed that the ionophoretic activity of PGB_x with Ca^{++} in lipid membranes was closely related to the Ca^{++} binding properties of PGB_x. The dissociation constant was found to be 0.5mM in the absence of KCl and that approximately 3 to 4 Ca^{++} molecules can be bound/PGB_x. These findings with the three different methods strongly suggests that the ionophoretic activity is directly proportioned to the formation of a PGB_x-Ca^{++} complex. These results would suggest the interaction of two carboxy groups on the PGB_x molecule for each Ca^{++}.

These studies demonstrate the importance of PGB_x binding of Ca^{++}. The results will be submitted for publication in the near future.

(5) <u>Ionophoretic Activity of PGB_x</u>: We reported in 1979 that PGB_x has ionophoretic activity, and is capable of moving Ca^{++} across naturally occuring membranes such as mitochondria and sarcoplasmic reticulum and to partition Ca^{++} between an aqueous and organic phase.

In late 1979 Weismmann and coworkers (op. cit.) confirmed the ionophoretic activity of PGB_x and characterized its ion selectivity as $Sr^{++} > Ba^{++} > Mn^{++} > Ca^{++} > Mg^{++}$.. These studies were conducted using multilamellar liposomes prepared from phospholipids in which a metallochrome indicator was encapsulated. They also confirmed that other prostaglandins did not show any measurable ionophoretic activity. We elected to expand on

these studies with liposomes as a means to evaluate the interaction of PGB_x and Ca^{++} and to determine the mechanism of ionophoretic activity. Measurements of Ca^{++} uptake can be determined by the measurement spectrophotometrically of the change in color of the encapsulated arsinazo III dye as Ca^{++} enters the phospholipid liposome. The rate of uptake can be determined and a kinetic analysis of ionophoretic activity can be determined.

The pH profile was determined and maximum activity observed at a pH of 6.9. It should be noted that this is close to the pK of 6.65 as determined by Mrs. E. Polis. It is of interest that the shape of the curve showed a maximum with decreasing activities at both higher and lower pHs. In comparison, the ionophores A23187 and x537A both have pH optima above their pK's but increasing the pH did not significantly decrease the ionophoretic activity. This would suggest that there is something rather unique about the activity of the PGB_x. A kinetic analysis indicated that the bacterial ionophore A23187 is approximately 1.4 orders of magnitude more active than PGB_x and approximately 1.8 orders of magnitude more active than x537A. Thus PGB_x falls somewhere between these two very active ionophores. From the slope of the lines of a double log plot of the initial velocity as a function of ionophore concentration suggests that two PGB_x molecules are involved in the movement of each Ca^{++} ion. This is also true for A23187 in that several investigators have proposed that the functional unit is a dimer. This data does not imply that there need to be one Ca^{++} bound for each PGB_x molecule but rather that two PGB_x molecules are involved in the move-

ment of a particular Ca^{++} ion.

Two possible mechanisms for movement of ions across membranes are that a molecule may serve as a mobile carrier or create a channel to permit the movement of the ions. Evaluation of the change in initial rate of Ca^{++} movement with increasing ionophore concentration suggests that PGB_x is serving as a mobile carrier in that the curve demonstrates saturation kinetics. In contrast deoxycholic acid did not demonstrate saturation kinetics and is known to cause a detergent effect on the membrane equivalent to formation of a channel at high concentrations. A study of the effect of temperature on ionophoretic activity also leads to a similar conclusion. The Arrenhenius plot reveals a direct dependence of ionophoretic activity on temperature, with increased rates of transport occurring at higher temperatures. The slope of the curve for both A23187 and PGB_x were distinctly steeper than that for deoxycholate which would suggest that PGB_x is a mobile carrier rather than a channel former.

The ionophoretic activities of some of the samples received for assay in the phosphorylating system were evaluated using the liposome system. A series of samples derived from an LH-20 column separation of crude PGB_x were evaluated. The results are reported in Figures 6 and 7 (the fractions were supplied by Dr. H. Shmukler and compared to a control batch of PGB_x.) The results indicate a direct correlation between oligomer size and ionophoretic activity. The larger the oligomer the more active the ionophoretic activity (LH-20 #1 is a larger MW than #2, etc.). As

can be noted, there appears to be some batch to batch variability in the samples in that the LH-20 #2 fraction tested was slightly different than the control PGB_x. Control PGB_x is supposed to be a sample of LH-20 #2. The methyl ester of PGB_x was found to have no activity in this system. In addition to PGB_x a series of ethyl analogues prepared by Dr. Nelson were also assayed. These fractions were also separated on an LH-20 column and as can be seen in Figure 8 for the bicarbonate soluble fractions and in Figure 9 for the bicarbonate insoluble fractions, the higher the molecular weight the more active the fraction was with respect to ionophoretic activity. The results are also summarized in Table #1 in which a direct comparison of the rate of ionophoretic activity in comparison to PGB_x is presented. It can be noted that there are several fractions of the ethyl analogue which appear to be more active than PGB_x. The liposome system will be employed as a standard assay procedure for samples in the future.

(6) <u>Perfused Heart Studies:</u> In an attempt to develop a whole organ system for evaluation of PGB_x it was decided to conduct preliminary experiments on the effect of PGB_x on a perfused rat heart preparation. A recirculating perfused rat heart system was established in which the rate of heart activity could be measured. With this system it was possible to insult the heart by stopping the perfusion and permitting the heart to become anoxic. Preliminary studies demonstrated that if perfusion stops for fifteen to forty-five minutes that the heart is unable to recover. In preliminary studies, the presence of PGB_x during the anoxic phase protected the heart from loss of function when the perfusion was

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reinitiated.   These results suggest that it will be possible to
use the perfused heart system as a means to evaluate the protect-
ive effect of PGB_x during anoxia in the intact tissue.   This ap-
proach opens a variety of new directions including the ability
to determine in a controlled series of experiments the changes
in mitochondrial function, structural changes and perhaps the
specific sites of action of PGB_x in the tissue.

Table 1

Activity of fractions of LH-20 chromatography of Ethyl Analogue prepared by Dr. G. Nelson (St. Josephs University). BS series are sodium bicarbonate soluble and N series are sodium bicarbonate insoluble. See Figure 1 and 6 for description of assay procedures for oxidative phosphorylation and ionophoretic assays.

SAMPLE	OXID. PHOSPHORYLATION		IONOPHORE	
	50% PROTECTION	%PGB$_X$	RATE*	%PGB$_X$
	μG/MG PROT.			
PGB$_X$	2.4 ± 0.4	(100)	30	(100)
ETA - BS 90	8.2	30	108	28
BSI 90	21.	10	16	187
BSII 90	5.7	42	28	107
BSIII 90	4.4	54	114	26
BSIV 90	11.8	20	> 120	< 20
BSV 90	11.4	20	> 120	< 20
ETA - N 90	23.6	10	95	32
NI 90	20.	12	40	75
NII 90	7.9	30	80	38
NIII 90	16.	15	> 320	< 10
NIV 90	> 25.	0	> 240	< 10

*RATE = μG SAMPLE/ML FOR A RATE OF $2\mu MCA^{++}$/MIN.

Figure 1. Assay for Protection of Mitochondria From Loss of
Oxidative Phosphorylation

Rat liver mitochondria, (8mg of protein) were preincubated
for 45 minutes at 30°c in 18mM KH_2PO_4-K_2HPO4,pH7.4, 10mM MOPS,
7mM Mg Cl_2, 36mM Ketoglutarate. After preincubation,hex-
akinose-glucose and 1.0mM ADP added and incubated for 30
minutes at 30°c. Where indicated as ATP control mitochondria
preincubated with 1.0mM ATP. Total volume was 2.8 ml.

EXP. VII-17

-22-
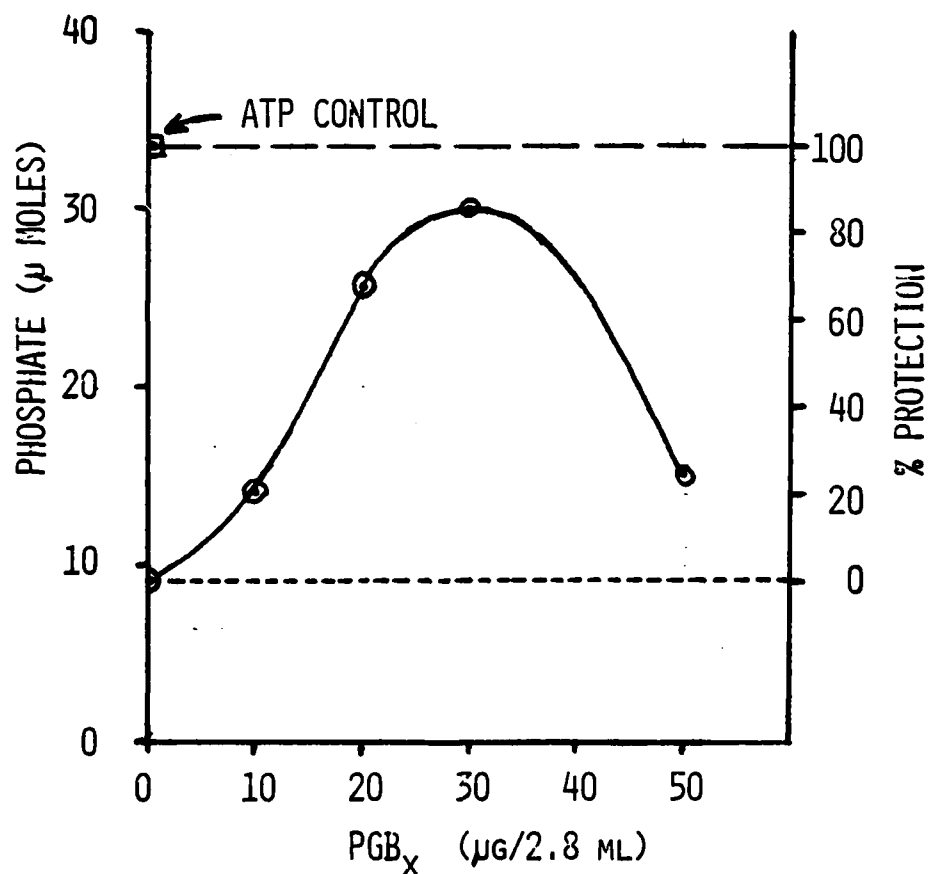
Figure 2. Dose Response Curve of PGBX on Oxidative Phosphorylation

Preincubation and incubation system as indicated in Figure 1; where indicated, no preincubation was conducted but reaction initiated at zero time.

△   NO PREINCUBATION

▣   ATP IN PREINCUBATION

◐   NO ATP IN PREINCUBATION
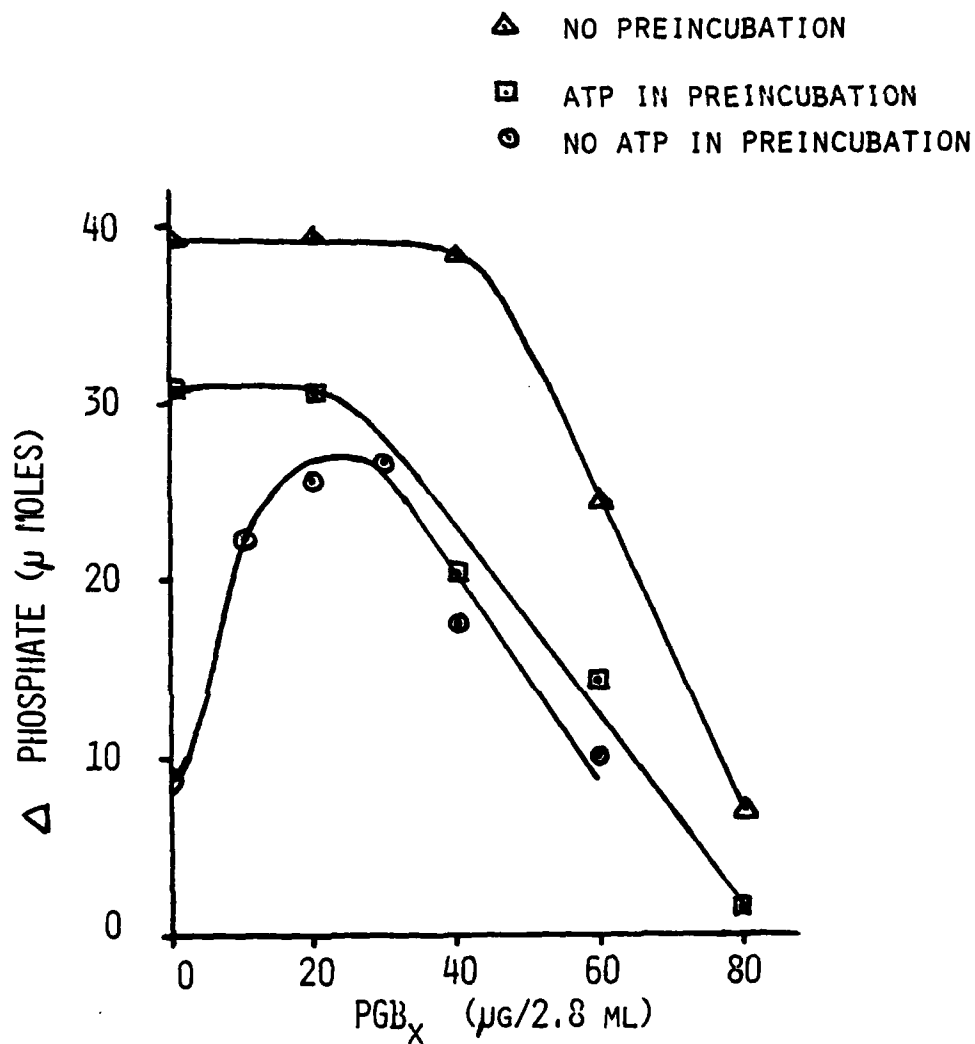
EXP VII 26

Figure 4. Effect of Bovine Serum Albumin on PGB_x Inhibition of Oxidative Phosphorylation.

     Assay system and incubation periods as in Figure 1. No preincubation period. Albumin concentration adjusted to have molar ratios with PGB_x concentrations indicated (Serum albumin MW= 68,000)
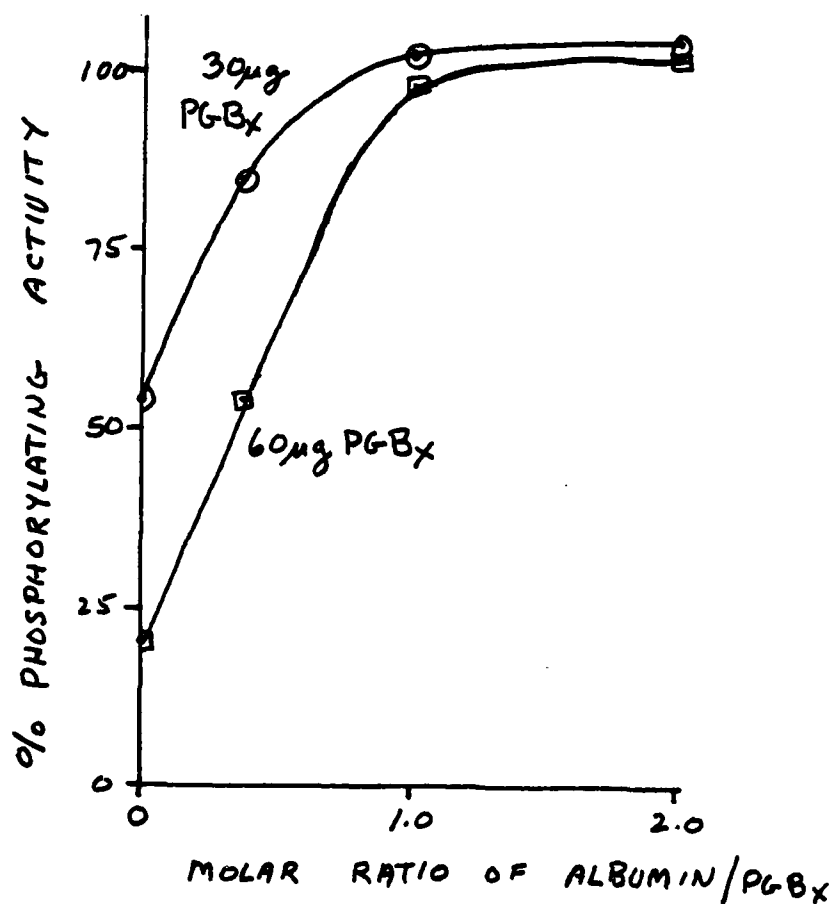
Figure 3. Effect of Varying Mitochondrial Content on Inhibition of Oxidative Phosphorylation by PGB_x.

Incubation system as described in Figure 1. No preincubation period. Values indicate mg mitochondrial protein in total volume of 2.8 ml. Incubation time, 30 minutes at 30°C.
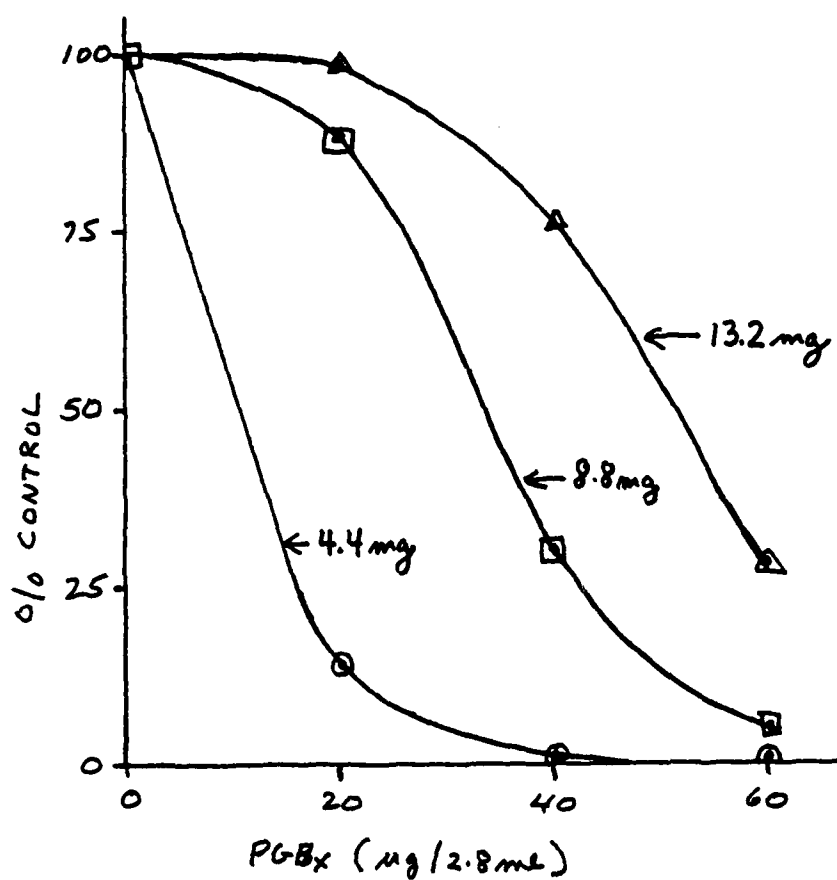
Figure 5

Submitochondrial particles were incubated 4 hours with (^{14}C) DCCD
(2 μg/mg protein) with no further additions. Equimolar unlabelled
DCCD or 30 μg PGB_x. Proteolipid was extracted with n-Butanol and
electrophoresed. Gels were either scanned for protein (280 nm) or
sliced into 4 mm fractions and counted.

Continuous tracing: protein
Bar graphs: radioactivity

Figure 5
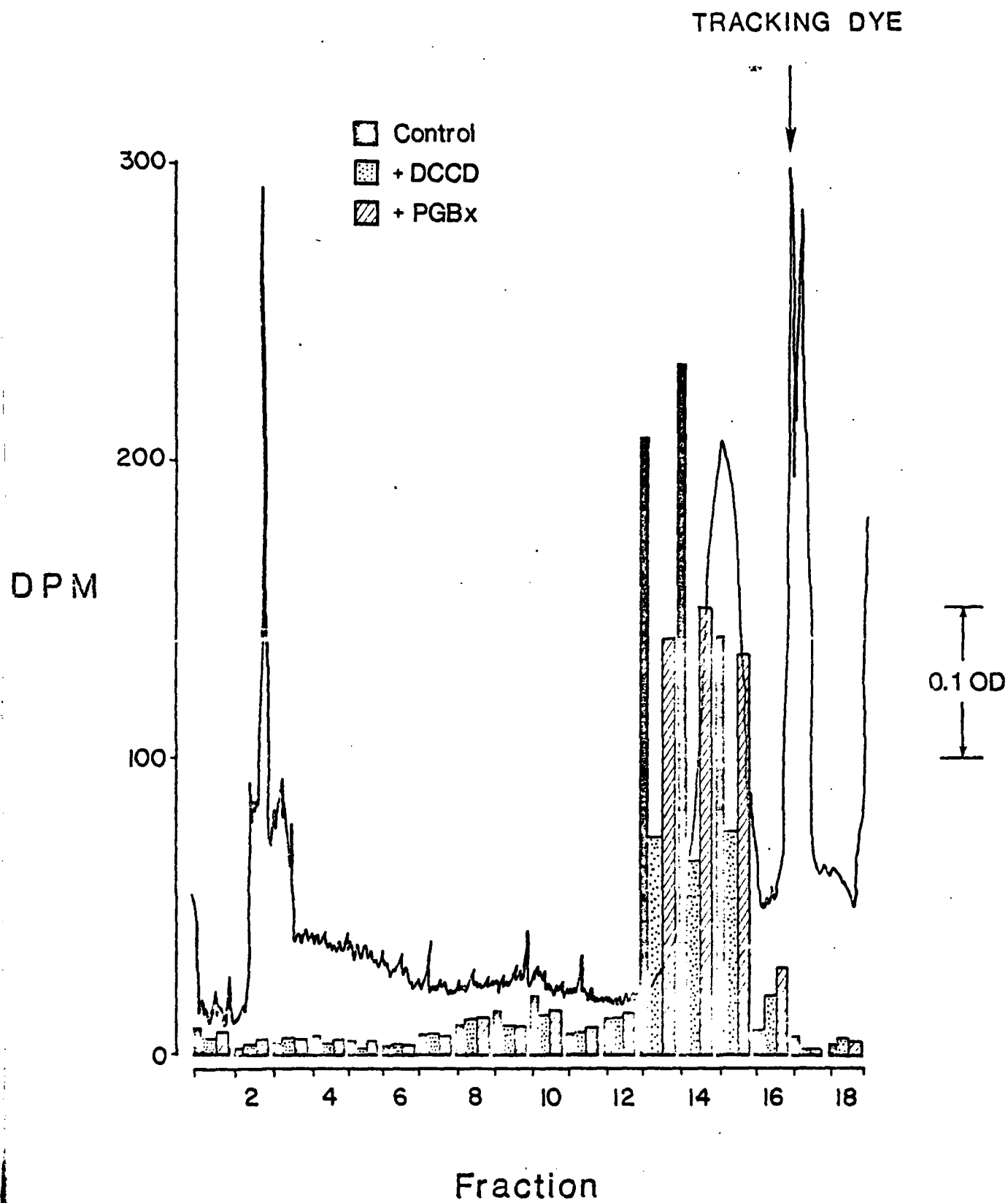
Figure 6: Ionophoretic activity of fractions of PGB_x separated by LG-20 chromatography.

Assay conducted with liposomes at pH 7.0. Ca^{++} added to external medium and rate of uptake as measured by changes in absorption of Arsenazo III encapsulated inside liposomes. Concentration of PGB_x expressed as $\mu g/ml$.

Figure 7: Ionophoretic activity of fractions of PGB_x separated by LH-20 chromatography.

Assay described in Figure 6. Concentration of PGB_x expressed as molarity. Molecular weights of fractions presented on page 39.

Figure 8: Ionophoretic activity of fractions of the Oligomerization of the Ethyl Analogue of PGB. Fractions are the sodium bicarbonate soluble fractions separated by LH-20 chromatography. Fractions ETA-B-IV 90 and ETA-B-V- 90 had no measurable activity at $60\mu g/ml$.

Figure 9: Ionophoretic activity of fractions of the Oligomerization of the Ethyl Analogue of PGB. Fractions are the sodium bicarbonate insoluble fractions separated by LH-20 chromatography.

Figure 6

-29-
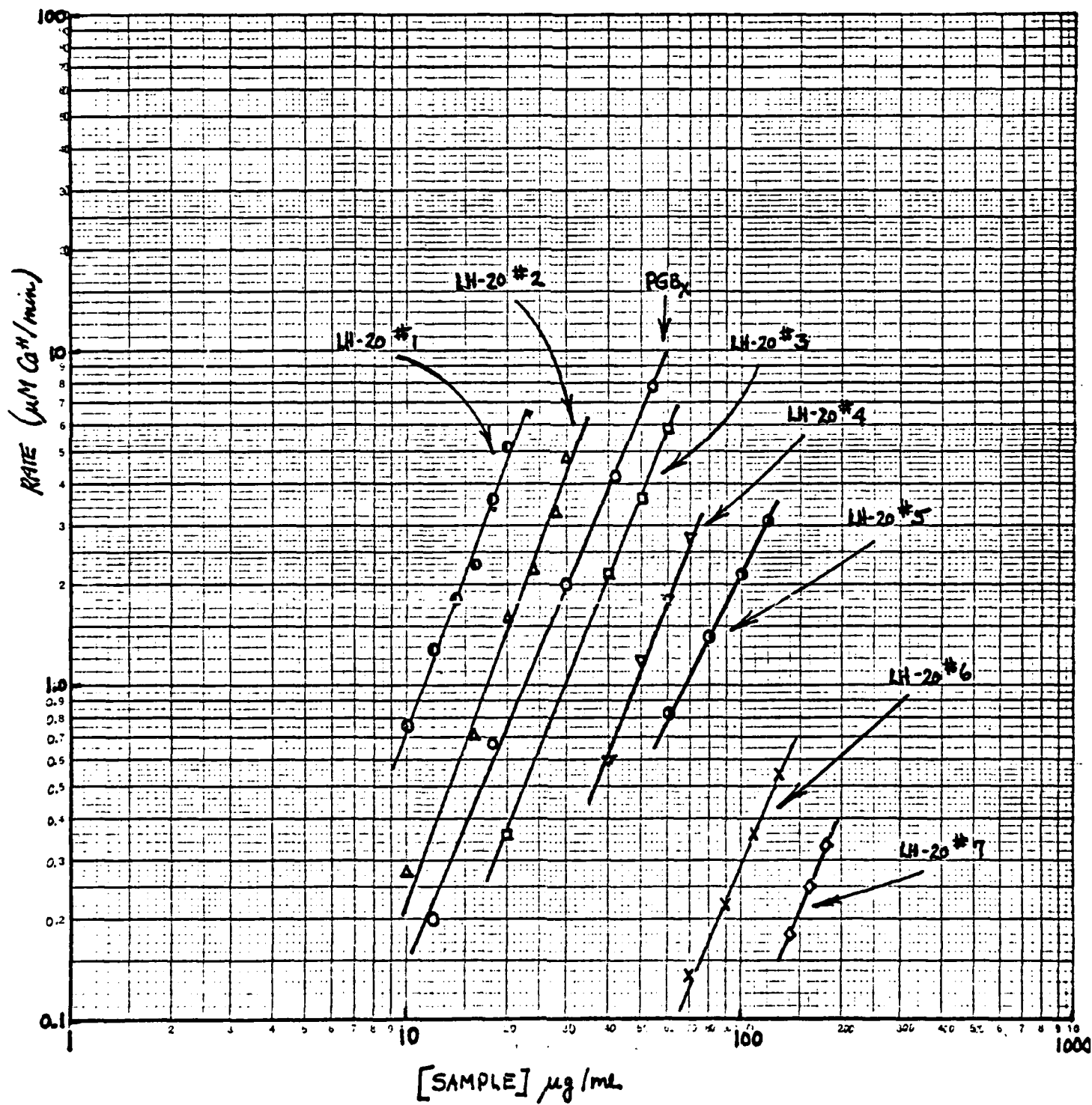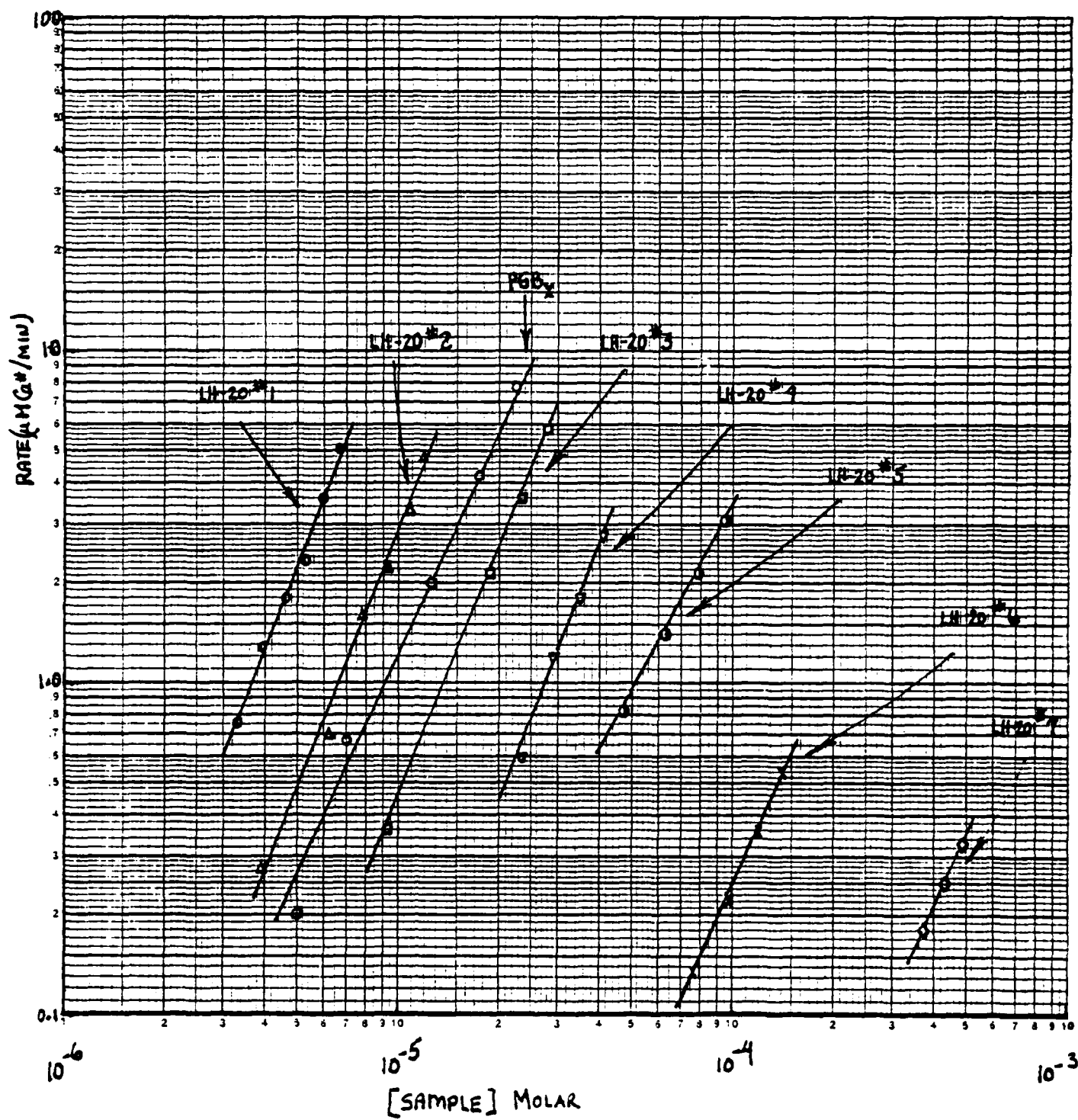
Figure 7
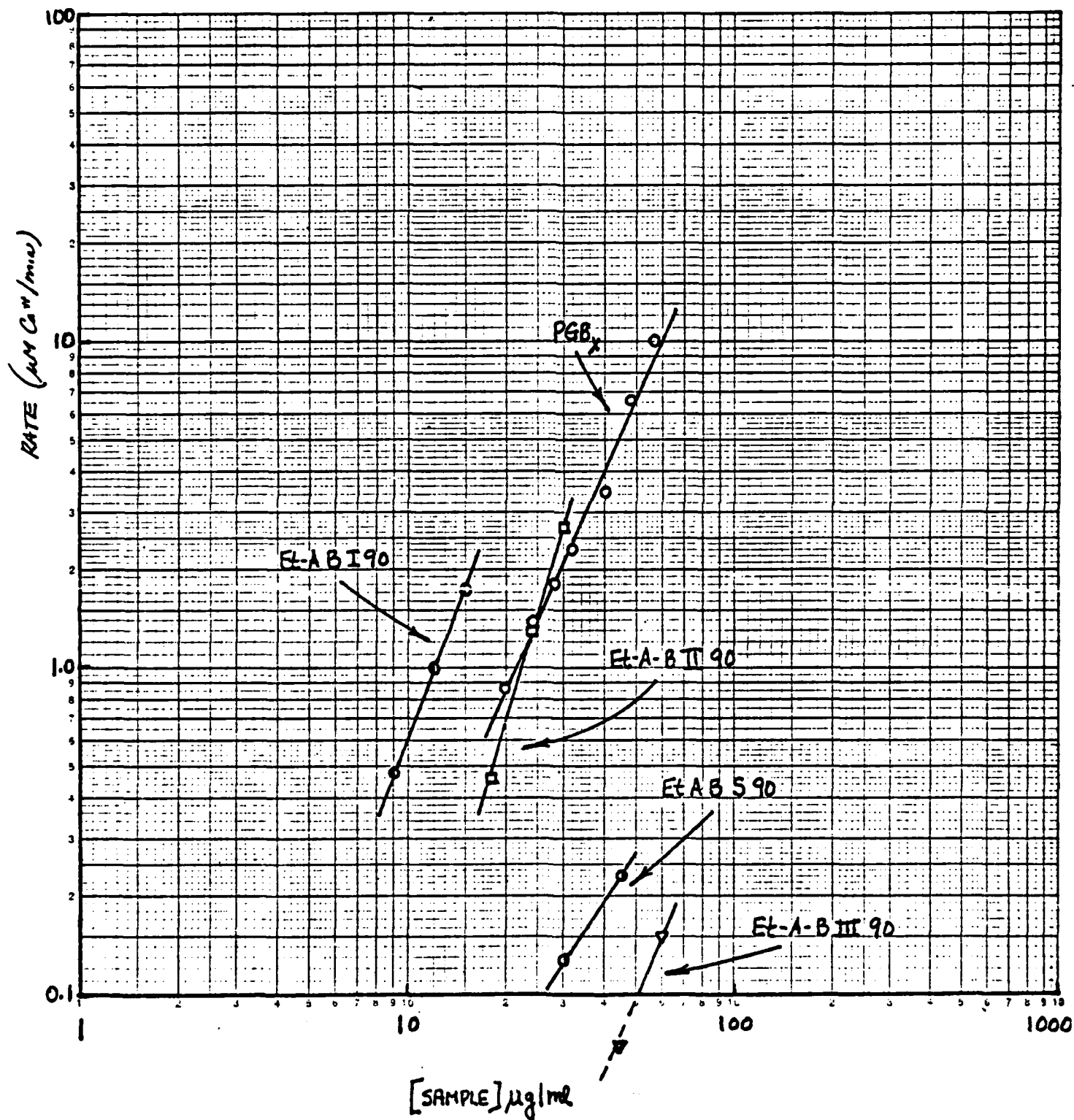
Figure 7

Figure 8

Figure 9
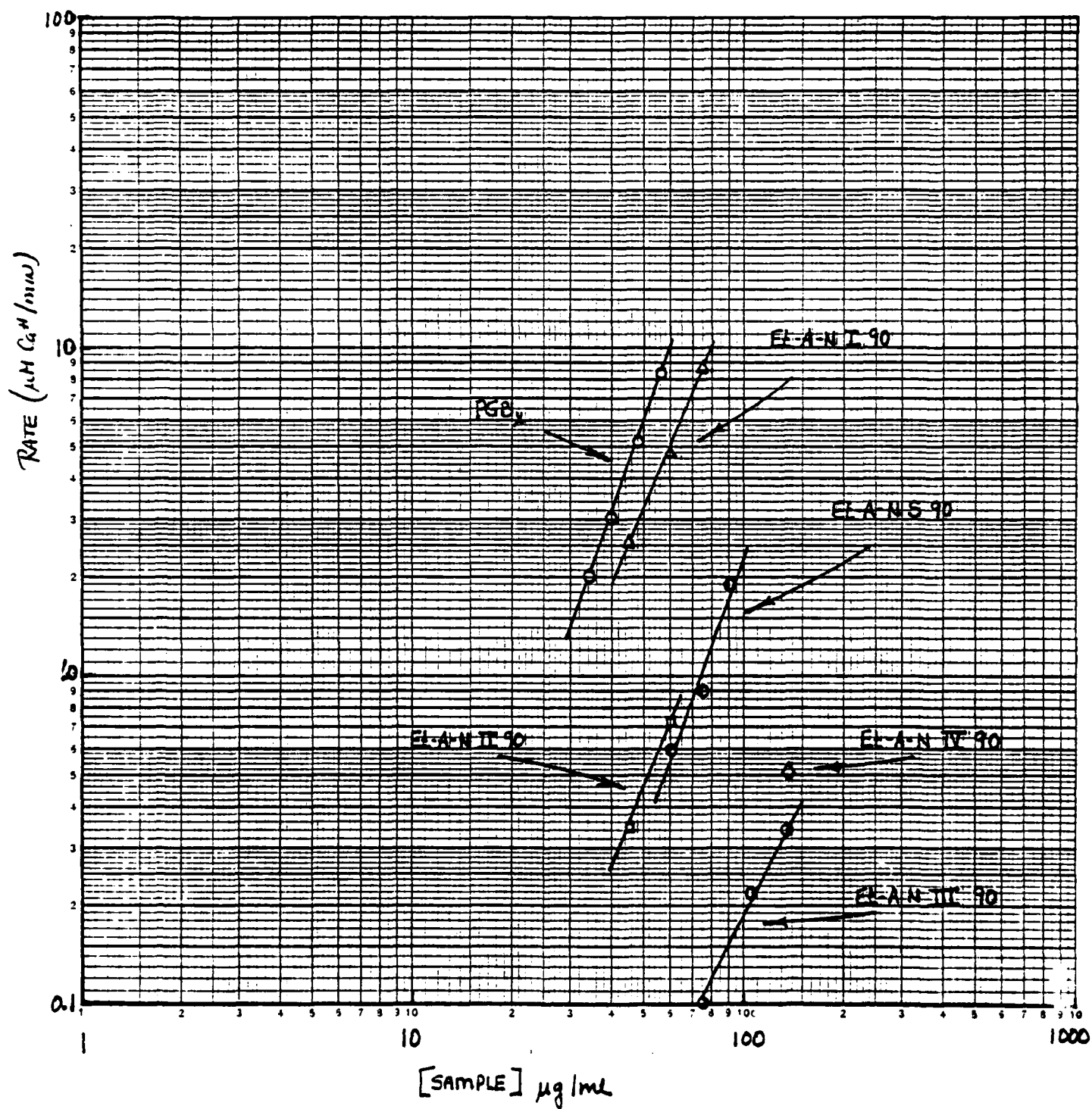
III. Report of the Research Program at NADC

I - <u>Preparation and purification of PGB$_x$</u>

A: Preparation of PGB$_x$ - Fiscal 1980

The details for the preparation of PGB$_x$ were worked out previously by Polis and Kwong[1] and improved by Shmukler[2]. In general this methodology is routine and the stockpiling of PGB$_x$ is on schedule. At the end of fiscal 1979 NAVAIRDEVCEN received 150 g of purified 15-keto PGB$_1$ from St. Joseph's University. This was converted to PGB$_x$ during fiscal 1980 to yield: 13.64 g of Type II and 10.0 g Type III both having <u>in vitro</u> mitochondrial effects equivalent to the standard PGB$_x$ normally shipped to ONR investigators. The present stockpiles of PGB$_x$ fractions are:

Type I	=	29.98 g
Type II	=	28.98 g
Type III	=	14.98 g
Type IV	=	31.0 g
Type V	=	39.55 g
Type VI	=	27.82 g
Type VII	=	6.18 g

43.96 g

By rechromatography of Type I and Type IV PGB$_x$ preparations on Sephadex LH-20 additional amounts of Type II and Type III PGB$_x$ may be obtained. Figure 1, panels 1 and 4 shows the "% distribution" of PGB$_x$ following rechromatography of Type I and Type IV PGB$_x$. Thus the recovery of Type II and Type III PGB$_x$ from Type I and Type IV should be about 16% and 73% respectively. On the basis the Type II and Type III

stockpile should be increased by 15.65 g and 10.9 g respectively.

B: Preparation of PGB_x precursors during fiscal 1979-1980.

In order to obtain homogeneous preparations of 15-keto-PGB_1 all precursors must be at least 99% pure. The synthesis of the 15-keto-PGB_1 precursors are carried out at St. Joseph's University and the intermediate stages are sent to NAVAIRDEVCEN for purification. For this purpose preparative chromatography of 20 g charges of intermediates are chromatographed on silica gel. The separated fractions are tested for identity and degree of purity by TLC, HPLC, NMR, UV absorption analysis, and if pure by these criteria are returned to St. Joseph's for the next synthetic step. During fiscal 1979 and 1980 NAVAIRDEVCEN received 1483 g of impure 7-(2-(3-hydroxy-1-octynyl)-5-oxocyclopentenyl heptanoic acid (compound #12 of NADC-78235-60)[1] from St. Joseph's. The purified product (63% yield) was returned to St. Joseph's for reduction to the cis-PGB_x. The cis-PGB_x received in this shipment was a 3 component system containing unreduced precursor, cis-PGB_x, and dihydro PGB_1. In previous years the precursor at this stage consisted primarily of cis-PGB_1 with small contamination with dihydro PGB_1. From this mixture the cis-PGB_1 could be isolated by preparative silica gel chromatography in one pass. However, the 3 component mixtures supplied this year could not be separated completely by silica gel chromatography even after repetitive chromatography. To obtain pure cis-PGB_1,

PVP chromatography had to be used and this reduced our productivity capability since only 2 grams of material could be processed in one day, compared to 40 grams per day on silica gel prep chromatography. The yield of pure cis-PGB_1 was 34%. The PGB_1 was isomerized at St. Joseph's and the resulting trans-isomer purified over silica gel to yield 317 g product (72%). The next step is the oxidation of the trans PGB_1 to 15-keto-PGB_1. At present an additional 94 g of compound #12 has been received from St. Joseph's and is in the synthetic scheme to yield additional trans-PGB_1. On the basis of this years experience 94 g of compound #12 should yield an additional 20 g trans-PGB_1 for a sum total of 337 g. On the basis of previous experience 337 g should yield about 174 g of 15-keto-PGB_1 which in turn should yield about 42 g of Type II and Type III PGB_x. Further rechromatography of Type I and Type IV should increase the yield by 32 g.

Summary of PGB_x stocks

Type II and III on hand	44 g
Type II and III fiscal 80-81	42 g
Rework of Type I and Type IV fiscal 80-81	66 g
Expected PGB_x on hand fiscal 80-81	152 g

II - <u>Further Purification of PGB$_x$</u>:

    A: Repetitive MEC on Sephadex LH-20

In previous studies it was found that rechromatography of the PGB$_x$ fractions separated by ME on Sephadex LH-20 increased the recovery of the Type II and Type III PGB$_x$ preparations. It was of interest then to rechromatograph a Type II preparation a number of times in order to purify the active principle and/or to obtain a PGB$_x$ preparation with a narrower molecular weight range of components than the standard Type II PGB$_x$. The separation methodology used in this study is summarized schematically in Figure 2. In this study 6620 mg crude extract of PGB$_x$, ie, the $NaHCO_3$ extract[1] were converted to the free acid, dissolved in methanol and chromatographed on Sephadex LH-20 as described by Polis <u>et al</u>[1]. The resulting 7 fractions were flash evaporated and analysed for weight recovery, $\bar{M}n$ and <u>in vitro</u> PGB$_x$ effects. These results are listed in Table I. As seen in this table, fraction 1-2*

DISTRIBUTION OF PGB$_x$ IN SEPHADEX LH-20 FRACTIONS: 1st MEC

Fraction	MW	Wt (mg)	K_a	Total K_a	K_i	Total K_i
1	3049	767	.82	629	1.18	905
2	2554	1107	.97	1074	1.33	1471
3	2137	825	1.02	842	1.48	1221
4	1706	706	.98	692	1.36	960
5	1257	1107	.47	520	.81	897
6	915	959	.33	316	.40	384
7	372	386	.30	116	.12	46
Sum		5858		4189		5885

Recovery %   88.5

Column Charge:
Crude Extract, 6620 mg

DISTRIBUTION OF PGB$_X$ IN SEPHADEX LH-20 FRACTIONS: 3rd MEC

Fraction	MW	Wt (mg)	K_a	Total K_a	K_i	Total K_i
1	2862	98.4	.87	85.6	.89	87.2
2	2364	209.1	1.18	246.7	1.18	246.3
3	2209	200.3	1.09	218.3	1.26	251.53
4	2008	115.5	1.06	122.4	1.23	161.8
5	1886	43.3	1.02	44.2	1.26	54.5
6	1466	22.9	.93	21.3	.91	20.8
7		2.43	.17	.4		
Sum		691.9		738.9		802.1
Recovery %		101.9		109.3		111.3

Column Charge:

2nd MEC #2 & #3, 679 mg

The overall weight recovery of PGB$_X$ in the 1st MEC was 88.5%. This suggests that 11.5% of the crude extract was either irreversibly adsorbed to the column, or adsorbed strongly to the column to elute outside the PGB$_X$ range. The overall weight recovery of the 2nd and 3rd MEC was approximately quantitative, ie, 95.7% and 101.9% respectively. This suggests that material not recovered in the 1st MEC is probably highly polymerized material tightly bound to the Sephadex LH-20 and probably not PGB$_X$.

Chromatography of the crude PGB$_X$ extract, 1st MEC, yields fractions varying in $\overline{M}n$ from 3049 to 372 as the retention time of the eluted material increased. Rechromatography of fractions 1-2 and 1-3 (2nd MEC) yielded fractions with $\overline{M}n$ varying from

2873 to 407. Even after 3rd MEC of fractions 2-2 and 2-3 the $\overline{M}n$ of the fractions varied from 2062-1466. Although the $\overline{M}n$ for fraction 3-7 could not be measured because of insufficient quantity, it would be reasonable to assume that the $\overline{M}n$ of fraction 3-7 would be similar to that of fraction 2-7.

The dry weights of all fractions separated in the repetitive MEC listed in Tables I, II and III were used to calculate the % distribution of PGB_x in each fraction in each separate MEC. These results are listed in Table IV. The % distribution of

% DISTRIBUTION OF PGB_x ON REPETITIVE
MEC ON SEPHADEX LH-20

Fraction	1st MEC	2nd MEC	3rd MEC
1	13.1	10.7	14.2
2	18.9	23.8	30.2
3	14.1	27.5	28.9
4	12.1	20.1	16.7
5	18.9	9.7	6.3
6	16.4	6.7	3.3
7	6.6	1.5	0.4

PGB_x in the 1st MEC is approximately evenly distributed between fractions 1 through 6. On rechromatography of fractions 1-2 and 1-3 over 70% of the PGB_x chromatographed appeared in fractions 2 through 4. On rechromatography of fractions 2-2 and 2-3 50% of the PGB_x was found in fractions 3-2 and 3-3.

The in vitro PGB_x assay data listed in Tables I, II and III show that the K_a was highest in fractions 2, 3 and 4 of the 1st MEC. Fraction 1-1 was about 80% pure while fractions

1-5, 1-6 and 1-7 were relatively impure. Rechromatography of fractions 1-2 and 1-3 (2nd MEC) showed that the K_a was equally spread throughout fractions 2-2 to 2-6 with fractions 2-1 and 2-7 exhibiting low values. When fractions 2-2 and 2-3 were rechromatographed (3rd MEC) only fraction 3-7 showed a low level of K_a. The results of the K_i distribution in the various fractions were similar to those found for the K_a distribution.

The UV absorption spectra, between $\lambda 200$ nm to $\lambda 400$ nm were measured for all fractions separated in this study. In general all fractions showed absorption maxima at $\lambda 243$ nm and absorption shoulders at $\lambda 300$-$\lambda 320$ nm. The major difference between the UV absorption spectra of the separated fractions was the increase in the $\lambda 300$-$\lambda 320$ nm absorption shoulder with increasing retention time of the fractions. The results are listed in Table V in terms of the ratio of the absorbance at

A_{243}/A_{310} OF FRACTIONS SEPARATED BY REPETITIVE MEC

Fraction	1st MEC	2nd MEC	3rd MEC
1	6.20	10.42	10.22
2	5.05	9.50	9.00
3	4.27	8.08	9.89
4	3.79	7.67	8.60
5	3.28	6.20	7.33
6	3.38	5.05	6.23
7	----	4.33	4.78

$\lambda 243$ nm to absorbance at $\lambda 310$ nm. A comparison of the

retention time and the A_{243}/A_{310} shows a progressive decrease
(or increase in A_{310}) with increasing retention time. A
comparison on the A_{243}/A_{310} of fractions with the same
retention time but successive MEC, ie, fractions 1-2 and
fraction 2-2, shows a marked increase in the ratio (or a
marked decrease in A_{310}). However, the data for the 2nd
and 3rd MEC showed no change in the A_{243}/A_{310} for fractions
with the same retention time.

The results of repetitive MEC of PGB_x on Sephadex LH-20
shows that this method does not yield homogeneous PGB_x pre-
parations as might have been expected. Instead the fractions
that were separated still appear to be hetereogeneous even
after 3 MEC. These results suggest that possibly even after
additional MEC of fractions 3-2 and 3-3 homogeneous preparations
of PGB_x would not be obtained. From these results it is obvious
that Type II PGB_x, the preparation currently supplied ONR
contractors for in vitro and in vivo animal studies, is a
highly complex mixture of oligomers varying in $\overline{M}n$ from over
2800 to below 400 daltons. It is interesting to note also
that the PGB_x fractions separated did not show a significant
increase in the specific activity of the PGB_x, ie, K_a.

One benefit realized with the repetitive MEC is that the
PGB_x fractions separated in MEC #3 must have a narrower range
of molecular weight components than that found in Type II
PGB_x. An additional advantage is the recovery of high K_a

activity in relatively low molecular weight fraction, e.g.
fraction 3-6 that had the following analytical values; $\bar{M}n$,
1466; K_a 0.93; K_i 0.92. It is conceivable that such a low
molecular weight preparation may be amenable to high re-
solution and/or field desorption mass spectral analysis.

   B: Fractionation of PGB_x by a combination of dialysis
       and MEC.

   In previous reports we showed that PGB_x preparations
could be separated into 2 distinct molecular weight fractions
by dialysis against dilute phosphate buffer at pH 6.85[3,4]
or by gel filtration on Sephadex G-100.[5] When the fractions
that were separated by dialysis were analysed by gel filtration,
each fraction showed two components. Thus, the dialysate
fraction showed an increase in a more retentive fraction
indicating low molecular weight, while the retentate showed
an increase in a less retentive fraction or high molecular
weight. From these experiments it appeared reasonable to
combine the techniques of dialysis and gel filtration in
order to fractionate PGB_x into mixtures of narrower molecular
weight ranges. This report decribes the separation of PGB_x in-
to 5 fractions with markedly different molecular weight ranges.
In addition, these fractions are characterized in terms of
in vitro PGB_x activity, UV absorption spectra, and molecular
weight.

Fractionation of PGB_x into Molecular Weight Groups:

1st step, aqueous dialysis: 2.3 g PGB_x, Type III[1] was
converted to the sodium salt and dissolved in 10 ml of 0.05 M
phosphate buffer pH 6.85.   This solution was dialyzed against
1000 ml of the same buffer using tubing with a nominal molecular
weight cut-off of 12,000.   Dialysis was carried out at 4°
for 24 hours with continual stirring of the external buffer.
At the end of this time the dialysate was replaced with fresh
buffer and dialysis continued for an additional 24 hours.
The dialysates were then combined, acidified to pH 3.0 with
dilute perchloric acid and shaken with 300 ml of ethyl acetate.
The phases were separated and the aqueous layer extracted 2
more times with ethyl acetate.   The combined ethyl acetate
extracts were washed 3-4 times with water and the water layer
discarded.   The combined ethyl acetate phases were flash
evaporated at 45° and the residue dissolved in ethanol.
This fraction was then converted to the sodium salt (yield
0.540 g).   The retentate was quantitatively removed from the
dialysis tubing and converted to the free acid (yield 1.81 g).

2nd step, aqueous dialysis 8000:   540 mg of dialysate from step
1 was dissolved in 5 ml 0.05 M phosphate buffer pH 7.1 and
dialyzed against 1000 ml of buffer using dialysis tubing with
a nominal molecular weight cut-off of 8000.   The dialysis and
the recovery of fractions was carried out as described under

"lst dialysis". The dialysate, designated "Fraction 1"
yielded 0.077 g while the retentate designated "Fraction 2"
yielded 0.403 g.

3rd step, methanol dialysis 12000: The retentate from the lst
step (1.81 g) was dissolved in 10 ml of methanol and dialyzed
against 1000 ml of methanol with one change of solvent after
24 hours. The dialysate and retentate were flash evaporated
separately to yield 1.692 g of dialysate and 0.040 g retentate
called "Fraction 5".

4th step, gel filtration: The dialysate from the 3rd step was
converted to the sodium salt and dissolved in 5 ml of 0.05 M
phosphate buffer, pH 6.85 and chromatographed on the preparative
AcA54 column at the rate of 350 mg per charge. Two fractions
were obtained as shown in Figure 3, which is a typical chromato-
gram of a preparative separation. The first peak was designated
"Fraction 4" and the second, "Fraction 3". The contents of the
fraction collector tubes comprising these fractions were combined
and concentrated by extraction into ethyl acetate at acid pH.
The ethyl acetate was flash evaporated and the residue dissolved
in ethanol and stored for later analysis. Figure 4 shows the
schematic flow sheet for the separation of PGB_x as described
above.

Properties of PGB_x Fractions:

In vitro PGB_x activity: All fractions separated in this study

were evaluated for PGB_x activity by methods reported previously.[1,5] The results are listed in Table VI.

THE IN VITRO PGB_x ACTIVITY OF PGB_x FRACTIONS SEPARATED
BY DIALYSIS AND GEL FILTRATION

Fraction	Wt (g)	%	K_a	K_i	Total K_a
PGB_x Type III	2.30		0.48	1.25	1.104
Separated Fractions					
1	0.077	3.35	0.23	0.25	0.018
2	0.403	17.52	0.64	0.67	0.258
3	0.460	20.00	0.95	1.08	0.437
4	1.190	51.74	0.56	1.21	0.666
5	0.040	1.74	0.57	0.61	0.023
Recovery	94%				125%

Molecular weights of PGB_x fractions: The $\overline{Mn}$ of each fraction separated in this study are listed in Table VII.

THE MOLECULAR WEIGHT AND POLYDISPERSITY OF PGB_X FRACTIONS

	Type III	Separated Fractions 1	2	3	4	5
MW (VPO)	1749	718	1700	1789	2061	2296
HPMEC						
MW_1	21402	--	26195	--	35607	36546
MW_2	3041	2622	1446	2630	2497	2563
% 1	10	--	3.5	--	10	17
% 2	90	100	96.5	100	90	83
$\bar{M}n$	4961	2622	2333	2630	5713	8335
$\bar{M}w$	11323	2622	11401	2630	22541	27872
$\bar{M}w/\bar{M}n$	2.28		4.89		3.95	3.34

The first line is the molecular weight of the PGB_X free acid measured by vapor pressure osmometry. The next part of the table lists the molecular weight of sodium PGB_X measured by MEC on Ultrogel AcA54 using sodium polystyrene sulfonates as standards for column calibration. Since a number of PGB_X fractions showed 2 components on analytical gel filtration the molecular weight of each component of the mixture, the percent composition of the mixture, the number average molecular weight ($\bar{M}n$), the weight average molecular weight ($\bar{M}w$) and polydispersity ratio ($\bar{M}w/\bar{M}n$) are listed in Table VII. The data show that the VPO molecular weights of these fractions range from 718 to 2296. The lowest molecular weight fraction had passed through

8000 d and 12,000 d dialysis tubing during aqueous dialysis, while the highest molecular weight fraction was retained by the dialysis tubing during both aqueous and methanol dialysis. Intermediate separations were realized by gel filtration of the dialysate from methanol filtration. Analytical MEC of these fractions show that fractions 1 and 3 are homogeneous and fractions 2, 4, 5 and the starting preparation contained two chromatographically separable species.

UV absorption spectra: The PGB_x fractions resolved in this study as well as the starting crude PGB_x were analysed for UV absorption spectra at a concentration of 0.03 mg/ml. The spectra were similar for all fractions in that they showed an absorption maximum at $\lambda 243$ nm and an absorption shoulder at $\lambda 290-310$ nm. The absorbance mg^{-1} cm^{-1} was calculated for each PGB_x fraction in the above characteristic spectral region and recorded in the Table below (VIII).

UV ABSORBANCE OF PGB_x FRACTIONS

Fraction	$A_{\lambda 243}^{mg/ml}$	$A_{\lambda 300}^{mg/ml}$	A243/A300
Crude PGB_x	28.33	4.00	7.08
1	25.83	5.00	5.17
2	29.07	5.27	5.52
3	32.17	4.33	7.43
4	31.67	4.03	7.86
5	29.77	5.10	5.84

The separation of Type III PGB_x described here yields a product that has a higher specific " in vitro activator" activity and a narrower molecular weight range than the starting preparation. Furthermore this purified fraction 3, is almost equivalent to Type II PGB_x from Sephadex LH-20 MEC^I in terms of specific "activator" and "inhibitor" activity.

The differences in the molecular weight of PGB_x fractions determined by VPO and MEC cannot be explained completely. One source of difference may be the lack of molecular weight standards of proper molecular structure similar to PGB_x. However, with this type of MEC calibrated with polystyrene sulfonates one would expect differences only on the order of 10-20%, rather than the 4 to 10 fold differences shown in Table II. The fact that VPO is carried out with the free acid in methanol, and MEC is carried out with the salt form in aqueous media, suggests that the latter method is complicated by aggregation of PGB_x to form larger molecules. Since all the fractions of PGB_x separated in this study, were concentrated by conversion of the salt to the free acid and back to the salt form before MEC, suggests that the molecular aggregates are of constant molecular weight rather than a non-specific aggregation. This is further born out by the fact that fractions 1 and 3 are homogeneous. Attempts to measure the

molecular weight of PGB_x salts by VPO measurement in aqueous

media were unsuccessful because of experimental difficulties.

It is important to note also that the biological effects of

PGB_x, both _in vivo_ and _in vitro_, are carried out in aqueous

media and that the specific molecular aggregation of PGB_x may

be important in its mode of biological action.

C: Newer Separation Procedures:

Chromatography on hydroxylapatite: Previous attempts in

this and other laboratories to purify PGB_x by adsorption

chromatography or by reverse phase chromatography have been

unsuccessful. One exception to this was silica TLC using

acetonitrile:ethyl acetate:methanol:0.1 N NH_4OH (14:6:2:5)

solvent system that separated 3 distinct fractions.[6] However,

because of the high alkalinity of the solvent system this

methodology could not be used for preparative purposes.

In the course of studies at NAVAIRDEVCEN that evaluated chro-

matographic adsorbents for use in the separation of PGB_x, it

was found that PGB_x could be separated into a number of

fractions by LC on hydroxylapatite using an increasing step

concentration gradient of phosphate buffer. The chromatographic

parameters were: hydroxylapatite (HTP) column 0.4 cm i.d. x 25 cm;

flow rate, 1.0 ml per min.; column temperature, 40°; phosphate

buffer pH 7.25 concentration gradient; 5×10^{-3} M, 10 min;

5×10^{-2} M, 10 minutes; 0.1 M, 10 minutes; 0.5 M, 10 minutes

and 10^{-4} M NaN_3, 20 minutes. In this report 25μg of PGB_x-Na Type III was injected for quantitative LC. Figure 5 shows the chromatogram of buffer only that shows the chromatographic anomalies associated with this system. In addition the column parameters are listed. Figure 6 shows the chromatogram of Type III PGB_x with unresolved peaks at 4.87 minutes, and resolved peaks at 14.98, 22.11 and 32 minutes. A % under each peak is the area % calculated by the recording integrator, which is a measure of the concentration of each separated component.

Preparative LC was carried out on columns 1.27 cm i.d. x 30 cm long at a flow-rate of 1.0 - 2.0 ml. The column was first equilibrated with water and then charged with 250 mg of PGB_x-Na Type III per run. The effluent stream was monitored with a UV monitor and separated into fractions when each chromatographic peak returned to base line. At this time the phosphate buffer concentration was increased and the fraction collected. All separated fractions were concentrated by acid extraction into ethyl acetate and flash evaporated. The separated fractions were analysed by analytical LC, recovery of weight, molecular weight, and in vitro mitochondrial activity to determine if this chromatography does indeed effect a separation of PGB_x.

Figures 7, 8, 9, 10, and 11 are analytical LC HTP chromatograms of fractions 1 through 5. Although the preparative

chromatography did not completely resolve the components, the enrichment of 4.99' component in fraction 1, the enrichment of component 15.64' in fraction 2, the enrichment of component 22' in fraction 3, 4 and 5 attests to efficacy of this separation procedure. The preparative procedure is summarized in Figure 12 which also shows the component distribution in the unfractionated PGB_x, the solvents used to elute the fractions and the distribution of components in each separated fraction. The results are summarized in Table IX.

HPLC - HYDROXYLAPATITE ANALYSIS OF PGB_x FRACTIONS
SEPARATED BY PREPARATIVE LC ON HYDROXYLAPATITE

PGB_x Fraction	Retention Time (Min)	4.9	15	22.3
Type III		3.27*	23.68	73.05
1		43.03	26.56	30.42
2		7.50	56.39	35.56
3		5.04	43.03	51.92
4		1.33	20.14	78.53
5		1.97	17.63	80.40

*Data listed as "Area %" of the three peaks indicated.

The weight of PGB_x recovery in each fraction and their corresponding molecular weights are listed in Table X.

Table X

THE WEIGHT AND MOLECULAR WEIGHT OF PGB_x FRACTIONS SEPARATED BY PREPARATIVE LC ON HYDROXYLAPATITE

PGB_x Fraction	Weight (mg)	%	MW
Type III	250		1919
1	4.7	1.9	681
2	13.1	5.2	1490
3	60.1	24.0	1672
4	34.4	13.8	1847
5	89.9	36.0	2289
Recovery	202.2	80.9	

Fractions 1 and 2 make up only a small amount of the total fraction while fractions 3 and 5 make up the majority of PGB_x recovered. In this chromatography only about 81% of starting PGB_x was recovered. Since the column packing did not appear to have any color it must be assumed then that the loss of PGB_x resulted during concentration and extraction steps. The molecular weight ($\bar{M}n$) of each fraction showed a progressive increase with increasing retention time. This suggests that, not only is adsorption chromatography involved here, but also some form of molecular exclusion. The results of in vitro mitochondrial PGB_x assay of each fraction is listed in Table XI.

Table XI

THE DISTRIBUTION OF PGB_x IN VITRO MITOCHONDRIAL
ACTIVITY IN FRACTIONS SEPARATED BY
PREPARATIVE HYDROXYLAPATITE LC

Fraction	Wt (mg)	K_a	Total K_a	K_i	Total K_i
Type III	250	.74	185	1.12	280
1	4.68	.23	1.08	--	--
2	13.09	.31	4.06	0.7	9.16
3	60.06	.71	42.6	0.79	47.45
4	34.33	.93	31.9	1.35	46.35
5	89.93	.78	70.1	1.45	130.40
Recovery (%)	80.8		80.8		83.3

These results show that fraction 4 exhibited a significant
increase in PGB_x activity over the Type III starting material,
while fractions 1 and 2 showed marked decreases in PGB_x
activity.

It may be concluded that the major application of hydroxyl-
apatite chromatography will be in the assessment of purity of
PGB_x fraction obtained by various separation methods. However,
its preparative use, although the results are not dramatic,
is not without merit.

III - Structure:

To date the structure of the active principle of PGB_x is still unknown even though this problem is being actively investigated in four different laboratories. From the repetitive chromatography and the molecular weight measurements it was concluded that PGB_x is a mixture of closely related oligomers. Molecular Weight of PGB_x. Previously Polis[14] reported that the molecular weight of PGB_x was 1600 calculated by MEC, and on this basis he postulated a tetramer structure for PGB_x. Since this M.W. value was calculated using standards structurally unrelated to PGB_x, this value was subject to considerable error. With the acquisition of a vapor pressure osmometor the M.W. of PGB_x could be determined more accurately and the value obtained for Type II PGB_x was 2300 d. Studies in this laboratory have shown that in aqueous solutions PGB_x aggregates to form high molecular weight polymers that could be separated from each other and to yield PGB_x preparations with different in vitro biological activity.[4] Since VPO measurements can only be made in organic solvents, this method gives the M.W. of free acid which apparently is unaggregated. In an attempt to gain corroborative evidence, the M.W. of PGB_x was measured by another procedure, ie, light scattering. M.W. of PGB_x Type II was measured with a laser beam low angle light scattering photometer and yielded a value of 9336 d. When this

sample was analysed by VPO the M.W. was 2418. Since the VPO method gives a number average M.W. ($\bar{M}n$) and light scattering gives a weight average M.W. ($\bar{M}w$), the ratio of these two values ($\bar{M}w/\bar{M}n$) gives a measure of polydispersity. In this case $9336/2418 = 4.07$. If polydispersity ratio were unity then the PGB_x would be considered homogeneous. Any value larger than one implies hetereogeneity. Since VPO measurements are weighted towards the lower M.W. while light scattering M.W. are weighted towards the higher M.W., then the values currently used must be considered as approximate values only. Obviously the true M.W. can only be reported for a homogeneous preparation.

Structural Studies by Physical Chemical Means:

PGB_x can be prepared by alkaline treatment of trans-PGB_1 (1) cis-PGB_1 (3), 13,14 dehydro-PGB_1 (4), 15-keto PGB_1 (2), PGE (5) and PGA_1 (6). (See Figure 20 for chemical structures). Under the conditions required for PGB_x formation PGE_1, PGA_1 and cis-PGB_1 are rapidly converted to PGB_1 and then to PGB_x. In contrast 13,14 dehydro PGB_1 (4) appears to undergo initial reaction by a different route.[12] In general PGB compounds without 13,14 unsaturation fail to oligomerize under the conditions for the formation of PGB_x.

In further studies Type II PGB_x was converted to methyl ester by treatment with diazomethane. The resulting product was devoid of PGB_x in vitro mitochondrial activity. Evidence for methylation only at the carboxyl function was provided

by ^{1}H and ^{13}C-NMR spectra. When the methyl ester was hydrolysed under mild alkaline conditions the PGB$_X$-free acid resulted and PGB$_X$ in vitro mitochondrial activity was restored.

The rapid disappearance of UV absorption at 296 nm, characteristic of the fully conjugated diendione system of 2,[13] concurrent with the appearance of a new absorption at 243 nm, results from base treatment (Figure 21). The change in UV absorption is consistent with the loss of unsaturation at carbons 13-14 to give a fully substituted cyclopentenone chromophore similar to that of 13,14-dihydro-15-dehydro-PGB$_1$ (8), a convenient model compound.

The loss of the 13,14-unsaturation is indicated in the IR spectra of PGB$_X$ as either the free acid or methyl ester (Figure 22). The loss is particularly evident in the conjugated double-bond stretch absorption of PGB$_X$ at 1630cm^{-1} as compared to the absorption at 1585cm^{-1} for 15-dehydro-PGB$_1$ (Figure 22A). The absorption at 1640cm^{-1} in 13,14-dihydro-15-dehydro-PGB$_1$ (8), associated with the fully substituted cyclopentenone functionality, is consistent with this interpretation.

The loss of the 13,14-double bond of 2 in PGB$_X$ formation is confirmed in the 360 MHz proton NMR spectrum of both PGB$_X$ and PGB$_X$ methyl ester (Figure 23). The appearance of the overall spectrum resembles the 13,14-dihydro-PGB skeleton as indicated by the similarity to the spectrum of 13,14-dihydro-

15-dehydro-PGB$_x$ (8). Integration of the -CH$_3$ and -OCH$_3$ absorption in PGB$_x$ methyl ester reveals a 1:1 relation that further indicates retention of the intact PGB skeleton in PGB$_x$. The general lack of resolution observed throughout the entire spectrum is consistent with a mixture of closely related oligomers. Spectra virtually identical to that of fraction 2 of PGB$_x$ were exhibited by all fractions with mito-chondrial activity obtained by Sephadex LH-20 exclusion chromatography. The same characteristic features exhibited in the ^{1}H NMR spectrum of fraction 2 were evident in all PGB$_x$ spectra regardless of the PGB precursor.

Further information concerning the general structural features of fraction 2 are provided by the ^{13}C NMR spectra (Figure 27). The loss of 13,14-unsaturation, with retention of the general skeletal features of the PGB system, is evident upon comparison of the ^{13}C spectrum of fraction 2 to the spectra of monomers 2 and 8. The unusual broadness of the absorptions of the sp^2 carbons associated with the cyclo-pentenone unsaturation is found in all fractions from Sephadex LH-20 chromatography exhibiting mitochondrial activity. This feature is also evident in the ^{13}C spectra of all PGB$_x$ regard-less of precursor. However, fractions can be isolated having this type of feature in the ^{13}C spectrum but without mito-chondrial activity.

Attempts were made to obtain the mass spectrum of PGB$_x$,

whether in the form of the free acid, methyl ester, or other derivatives, by application of a variety of techniques including field desorption and chemical ionization. These efforts were unsuccessful because of lack of volatility. However, the mass spectra of monomers 2 and 8 as well as dimeric reaction intermediates were readily determined under the same conditions that proved unsuccessful for PGB_x.

The detailed structural assignment is severely complicated by unavailability of a single pure component possessing mitochondrial activity even after extensive separation attempts. The problem is compounded by lack of exact molecular weight measurement and related information normally available from the mass spectrum.

Formation of the oligomers does appear to proceed through reaction at the 13,14-double bond of 2 with the retention of the fully substituted cyclopentenone system in the oligomer. Additional support is provided by the failure of compounds 7 and 8, which lack the 13,14-unsaturation, to undergo oligomerization under conditions of PGB_x formation. Oligomer formation by addition to the 13,14-double bond would result in generation of a number of chiral centers. Because of this, PGB_x in the molecular weight range of 2200-2800, containing oligomers with varying numbers of units, is probably further complicated by the presence of closely related stereoisomers. The general lack of resolution observed

in the ^{1}H and ^{13}C NMR spectra and the particular broadening observed for the sp^2 carbons associated with the cyclopentenone conjugation appear to be most consistent with a complex oligomeric mixture of closely related compounds. The loss of activity resulting from methylation of PGB$_x$ might be evidence for the essential nature of the carboxyl functionality for mitochondrial activity. Methylation of PGB$_x$ results in no major change in functionality since no loss in activity or change in structure occurs when PGB$_x$ is converted to the methyl ester and then hydrolyzed back to the free acid.

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Figure 1

Rechromatography of PGB$_x$ Types on Sephadex LH-20

Molecular Weight
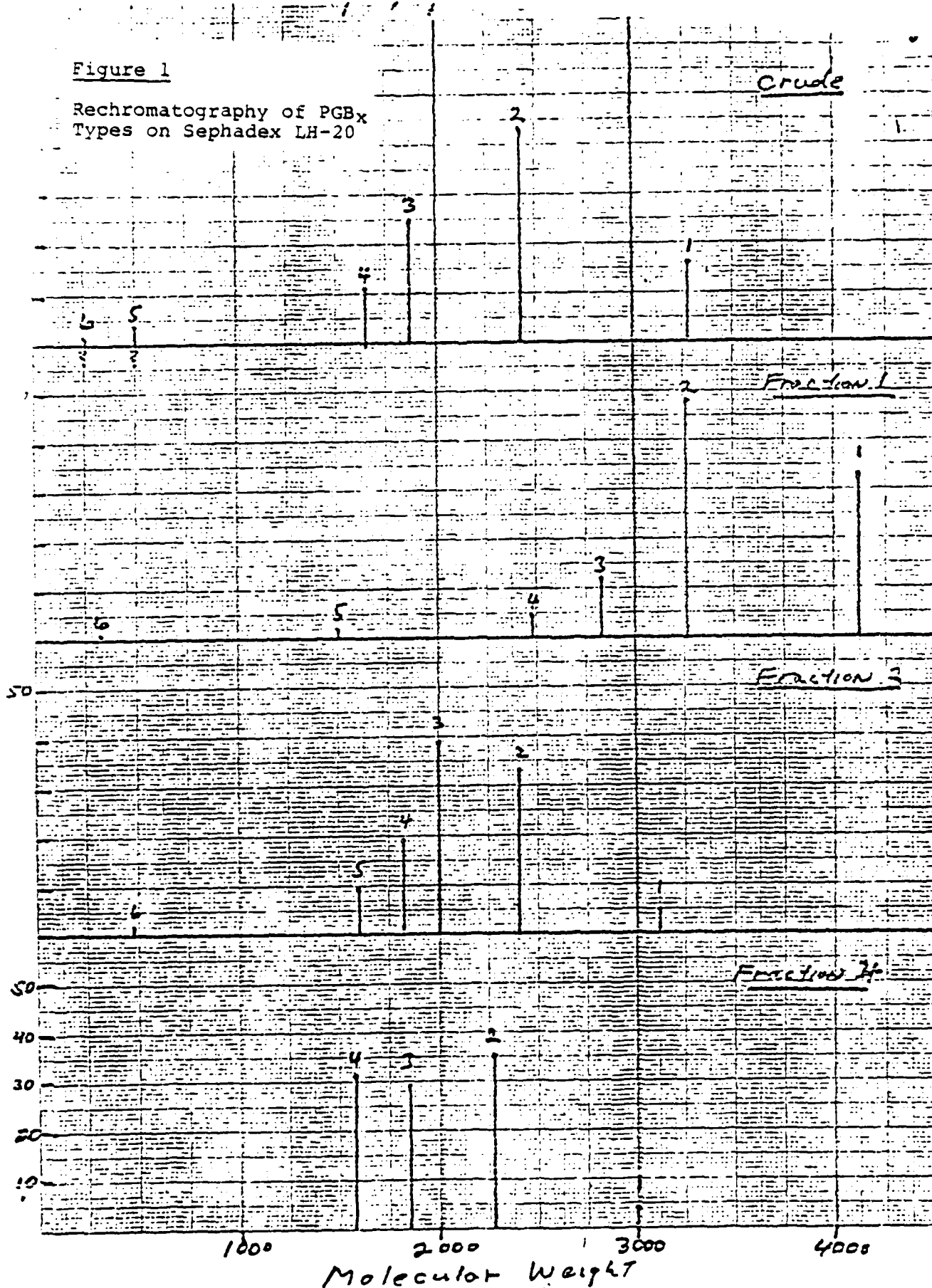
Figure 2

SCHEMATIC FOR REPETITIVE MEC OF PGB_X

ON SEPHADEX LH-20
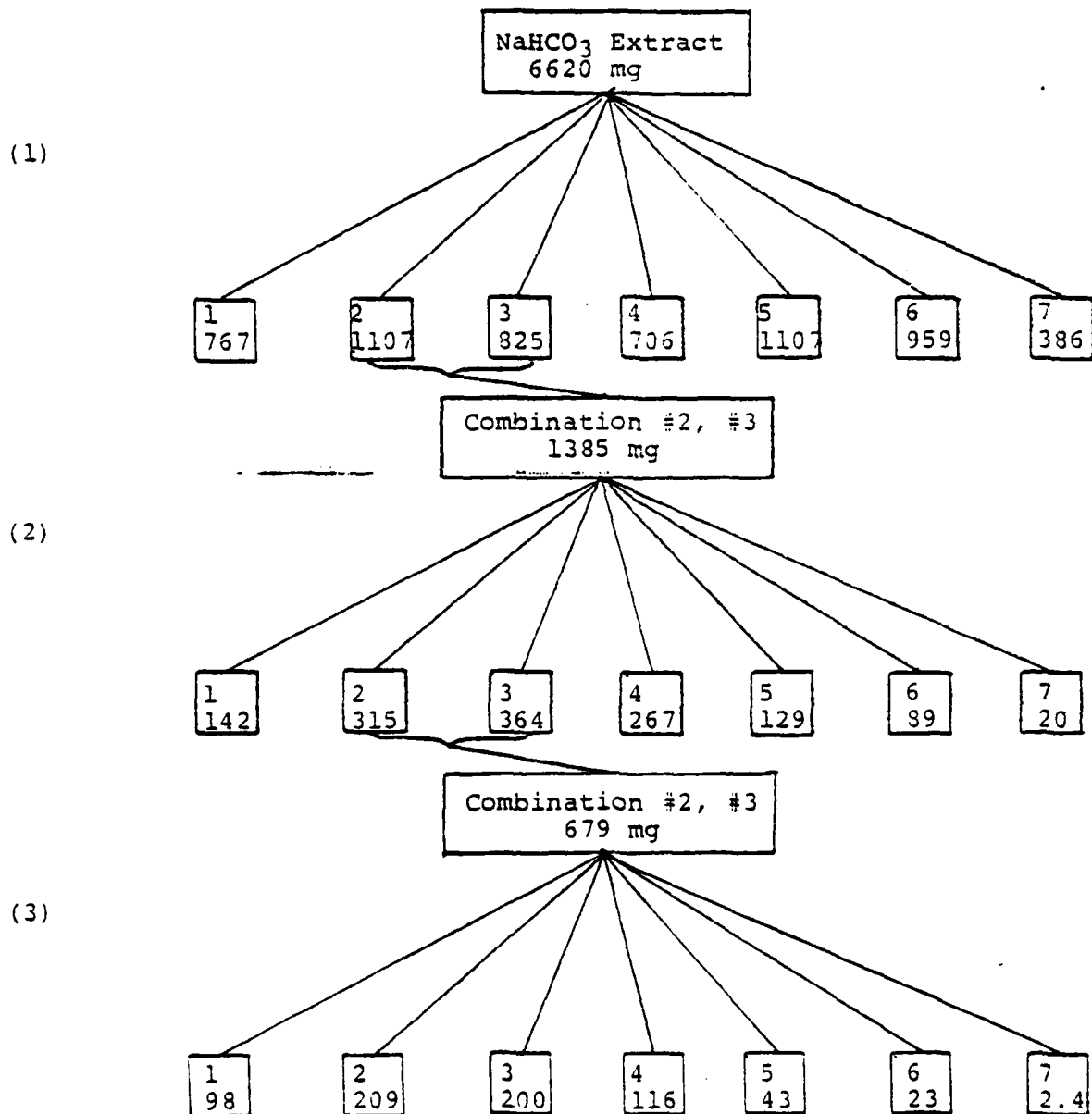
Figure 3:

Preparative MEC of PGB_x Type III on Ultrogel AcA54. Chromatographic parameters: column size, 2.54 cm i.d. x 40 cm long; carrier buffer, 0.05 M phosphate buffer pH 6.85; flow-rate, 1.0 ml per minute; fraction collector, 4 minute fractions; detector attenuation, 1.86 AUFS; detector path length, 0.05 cm; temperature, room ambient; maximum PGB_x sample, 250 mg; axis of ordinate, recorder chart scale divisions.
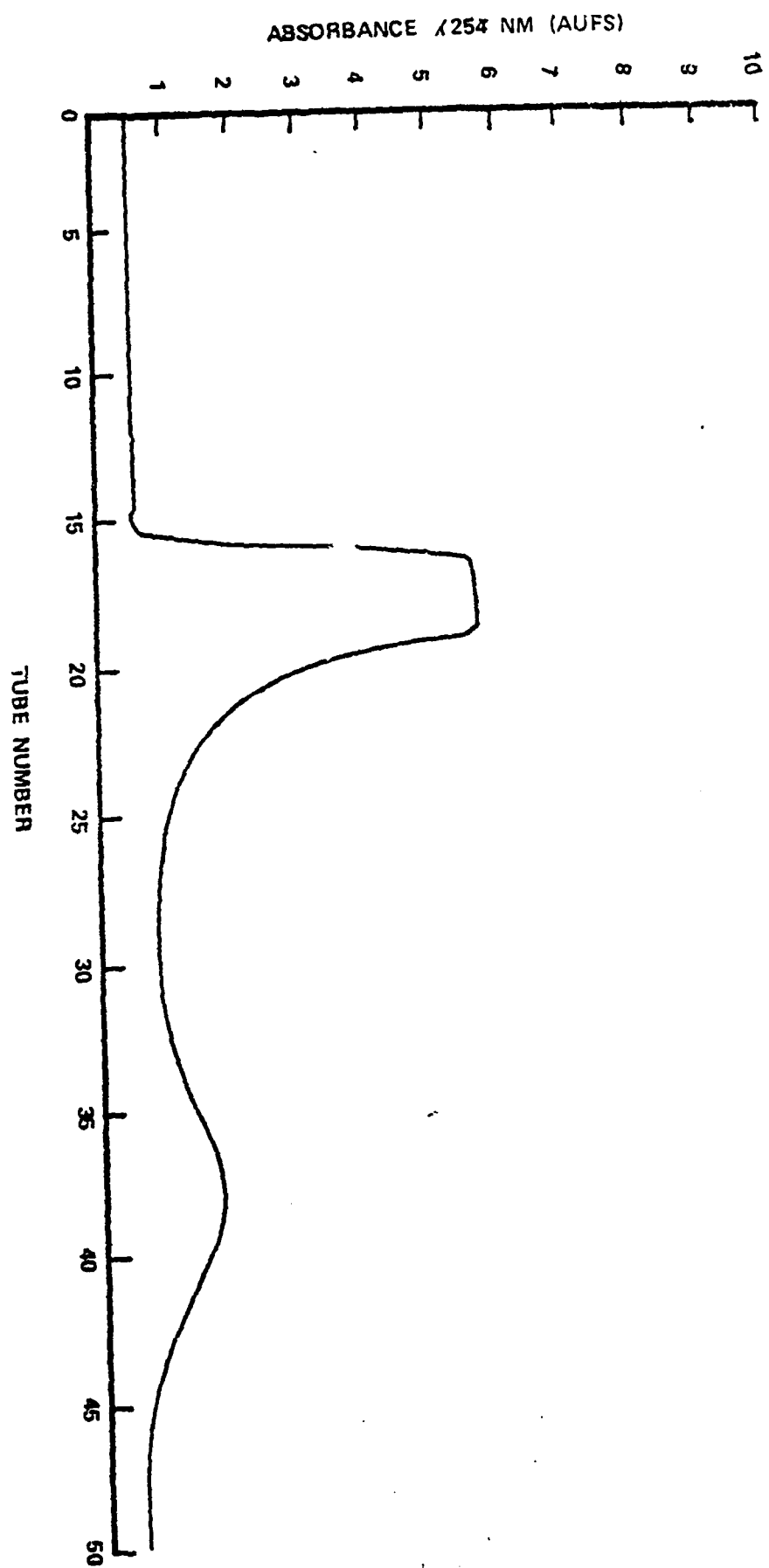
Figure 4

Schematic flow-sheet of separation procedure used in this study.

PGB$_x$ (FRACTION III)

DIALYSED VS PHOSPHATE BUFFER – 12000 d

RETENTATE
DIALYSED VS METHANOL

RETENTATE
FRACTION 5

DIALYSATE
M.E.C.

LOW RETENTION VOLUME
FRACTION 4

HIGH RETENTION VOLUME
FRACTION 3

DIALYSATE
DIALYSED VS PHOSPHATE BUFFER – 8000 d

RETENTATE
FRACTION 2

DIALYSATE
FRACTION 1

-67-
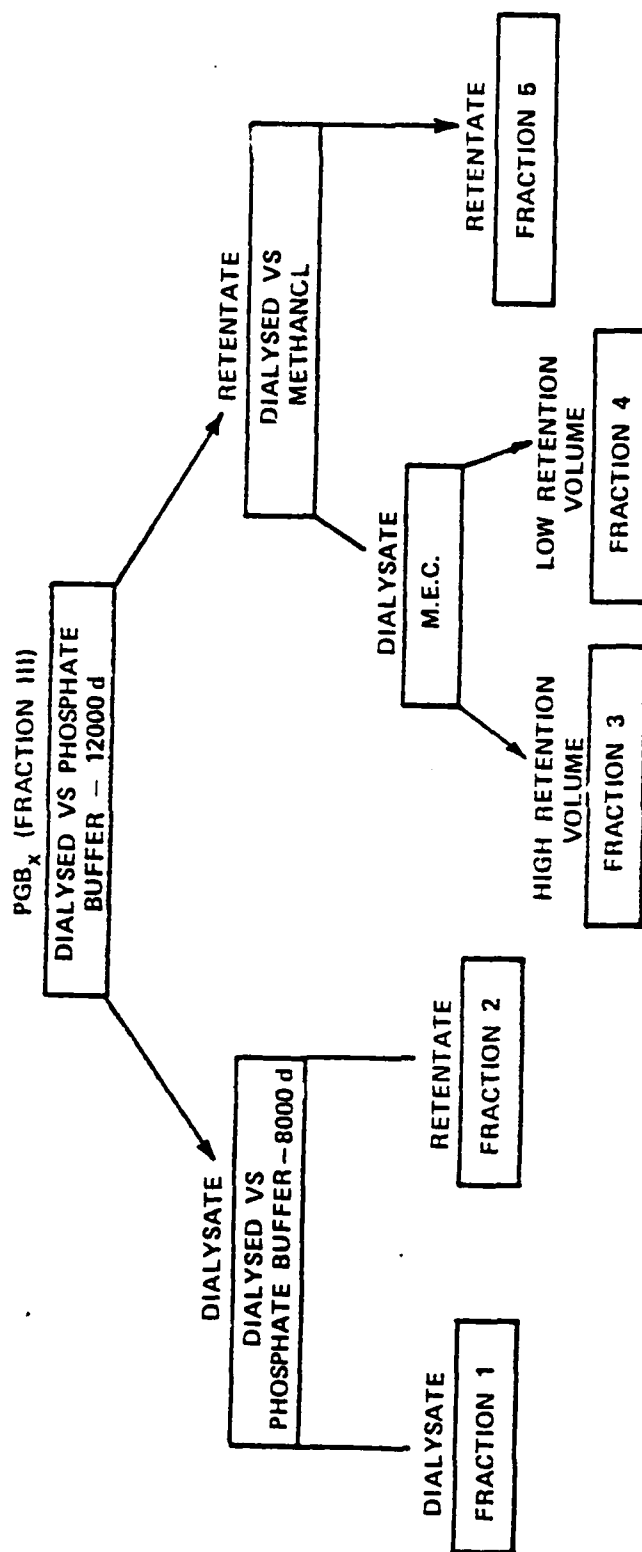
Figure 5:

HTP LC of buffer only.

Figure 5   079

ANALYTICAL HTP LC

Column: Biorad Hydroxylapatite HTP
         (¼" o.d. x 25 cm L)
Detector: λ 254 nm
Sensitivity: 1.0
Integrator Attenuation: 16
Flow rate: 1.0 ml/min
Chart speed: 0.5 cm/min
Solvent: Phosphate Buffer, pH 7.25
         (10^{-4} M NaN$_2$)

39.·98

Blank

0.005M   0.05 M   0.1M   0.5M   H$_2$O

10   20   30   40   60

MINUTES

A λ 254 nm
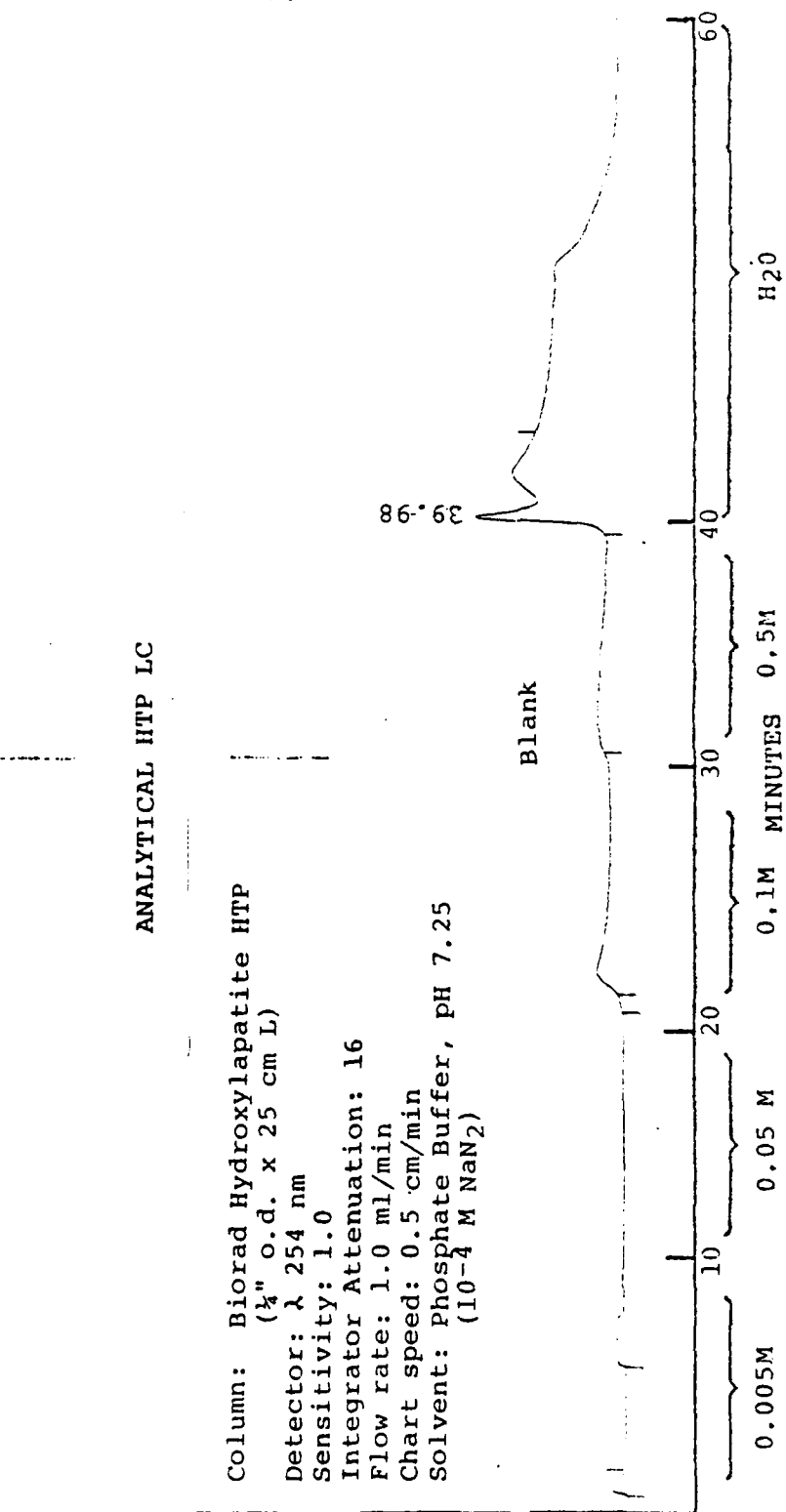
Figure 6

ANALYTICAL HTP LC

PGB$_x$ Type III

A% = 73.0

A% = 23.7

A% = 3.3
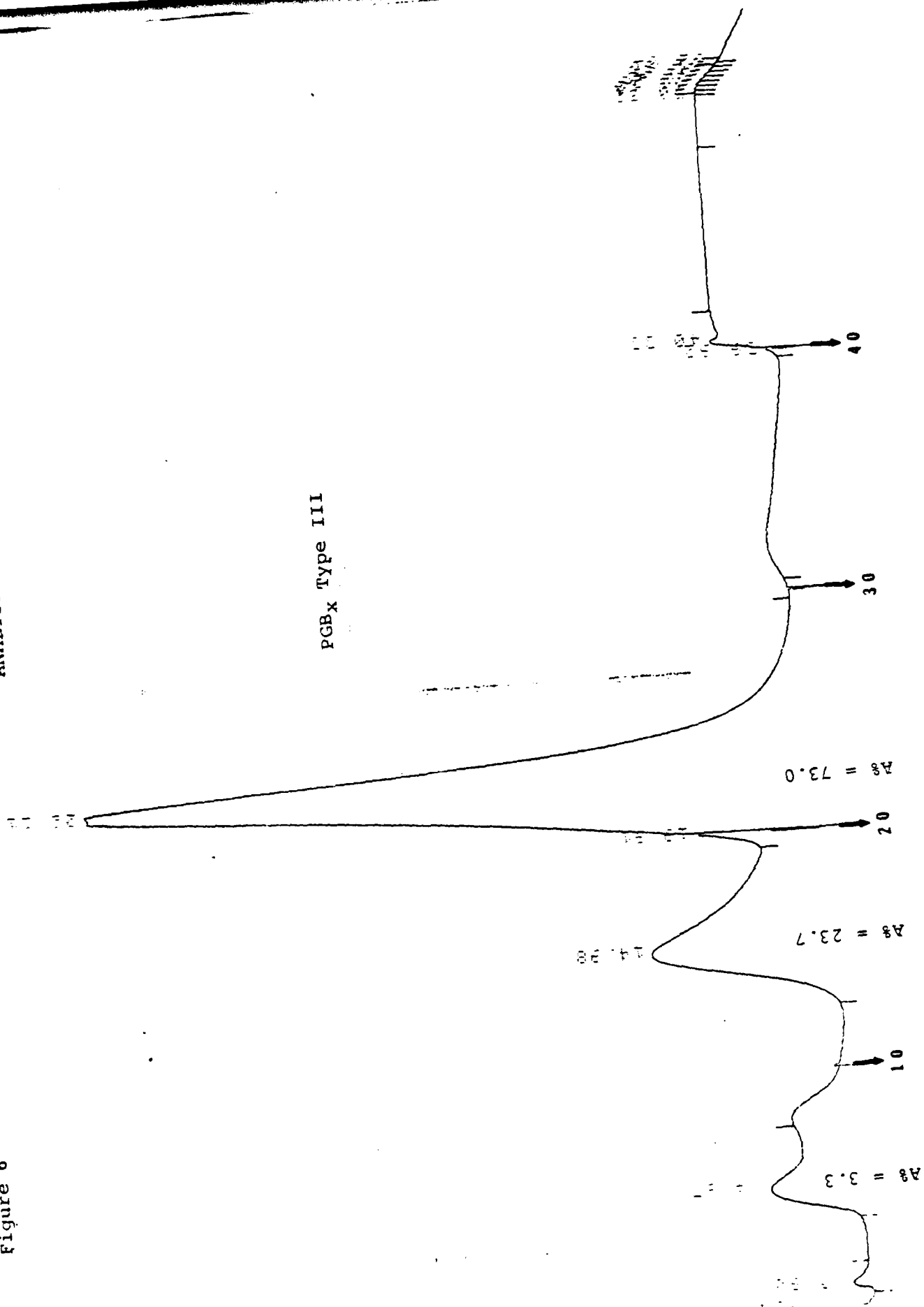
Figure 7

ANALYTICAL HTP LC

PREP. HTP FRAC. 1

A% = 43

A% = 27

A% - 30
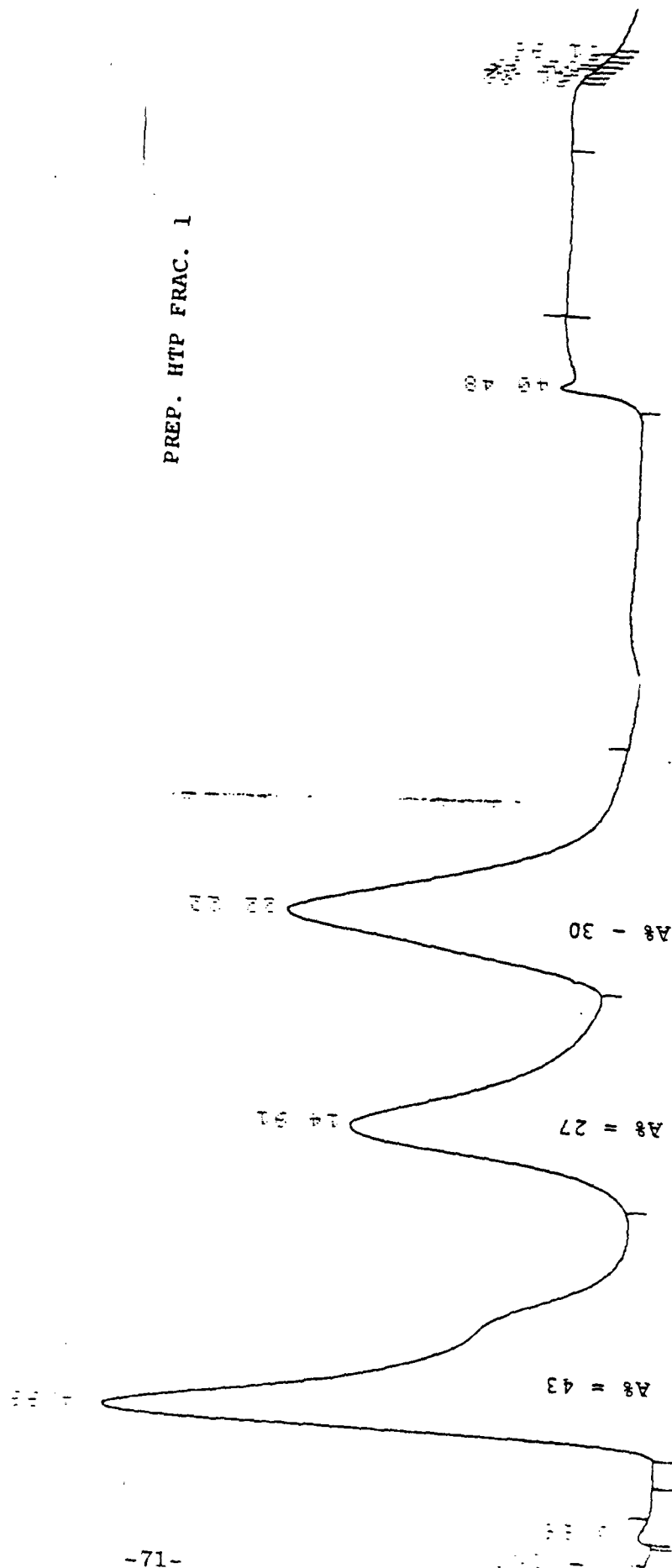
Figure 8

DET _A1A Col 1900_ SPEED_
SAMPLE_
SAMPLE CONC. _1 mg/ml_   INJ. VOL. _25 μl_

ANALYTICAL HTP LC

PREP. HTP FRAC. 2

A% = 7.6

A% = 56.7

A% = 35.7
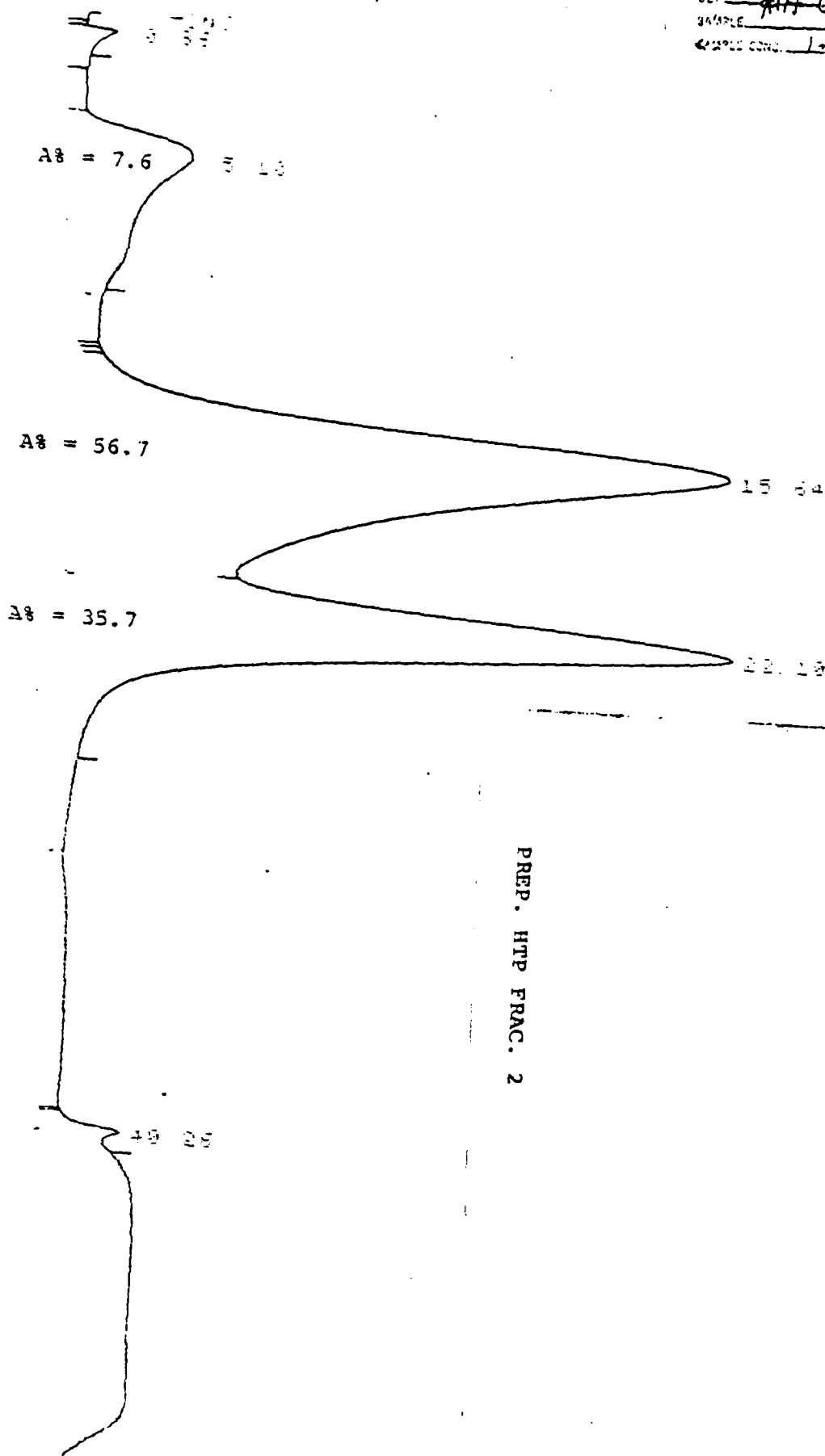
Figure 9

ANALYTICAL HTP LC

Prep. HTP Frac. 3

Figure 10

ANALYTICAL HTP LC

Prep. HTP Frac. 4

Figure 11

A% = 2.0

A% = 17.6   15.73

A% = 80.4   21.57
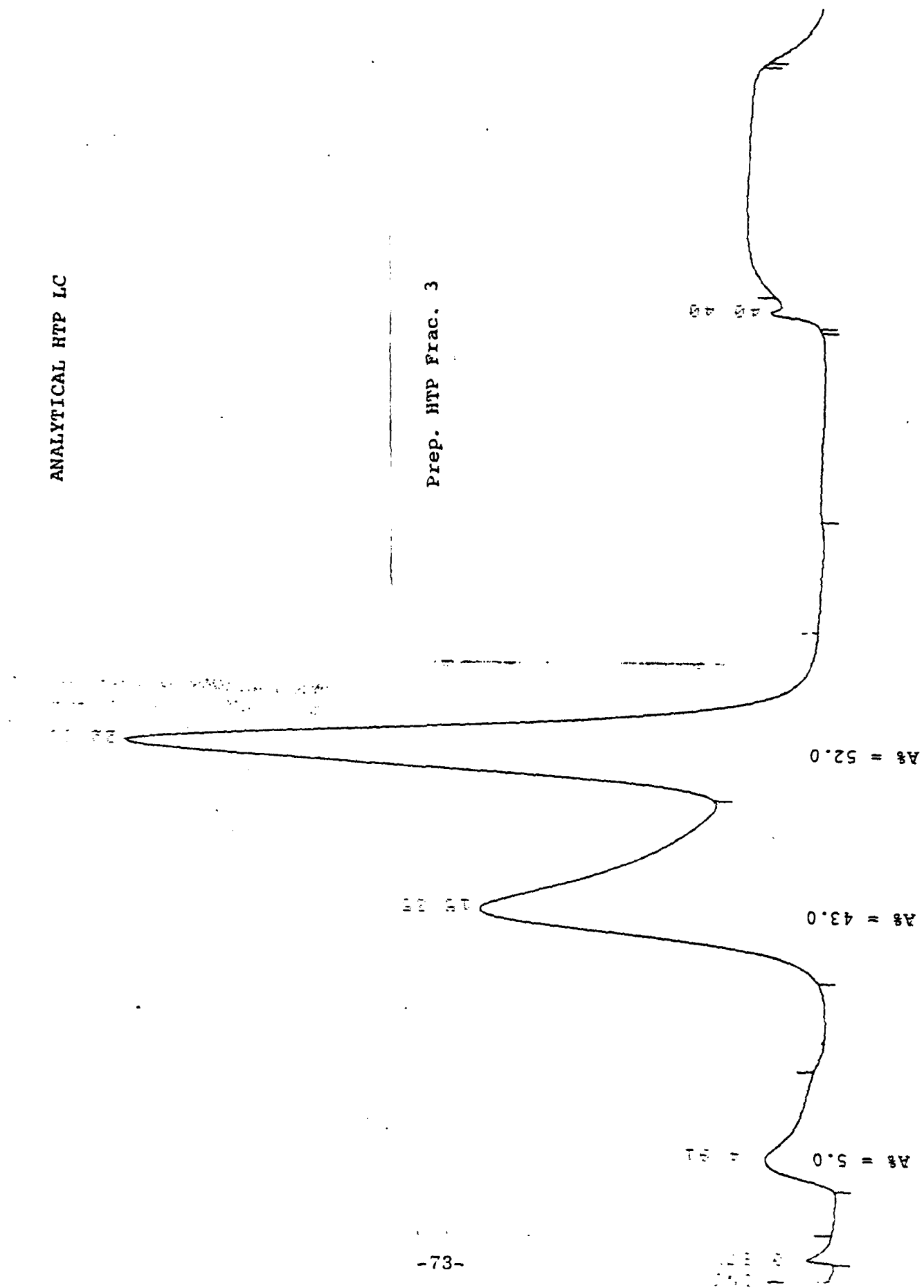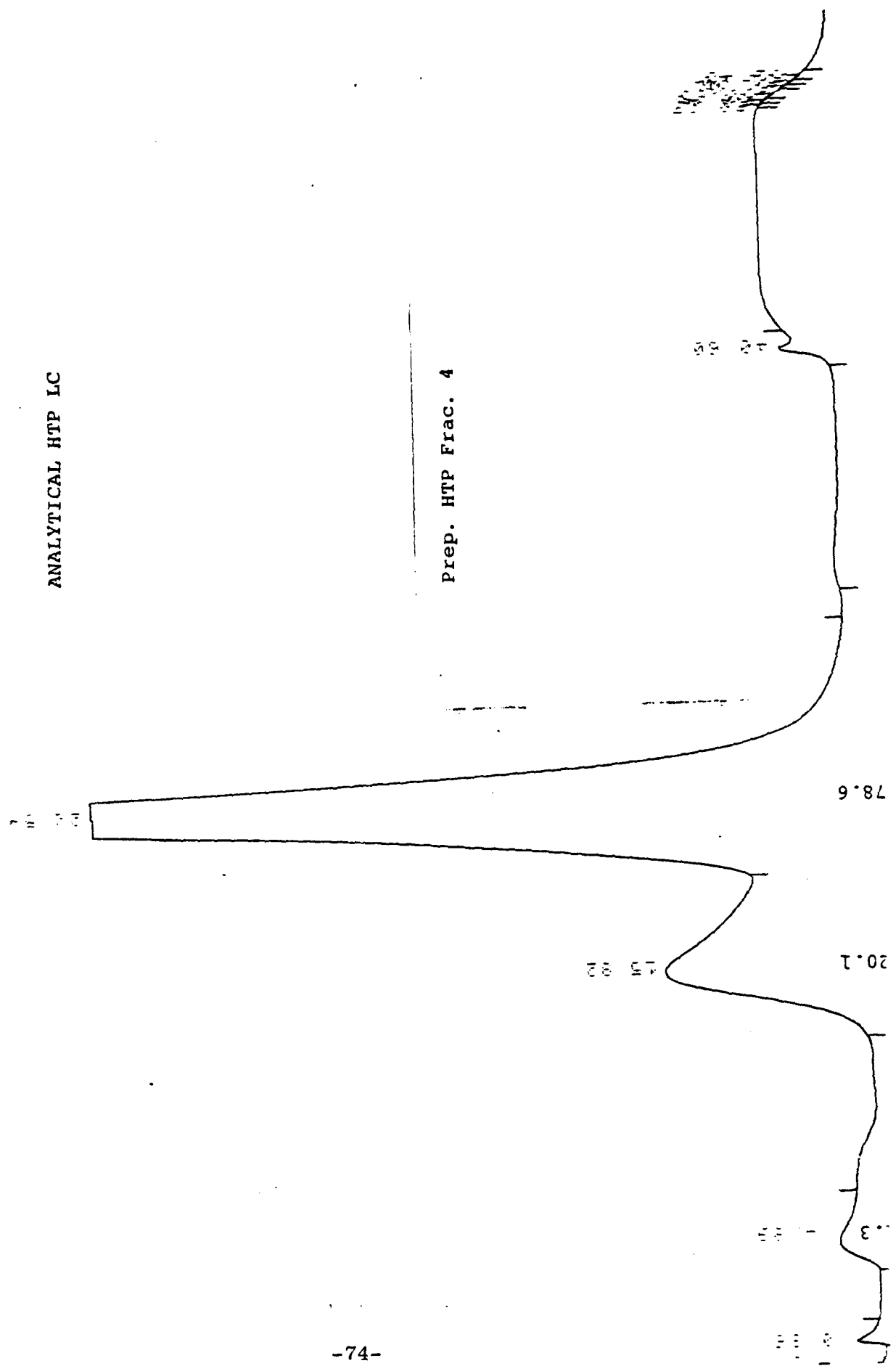
ANALYTICAL HTP LC

Prep. HTP Frac. 5

10.30

75
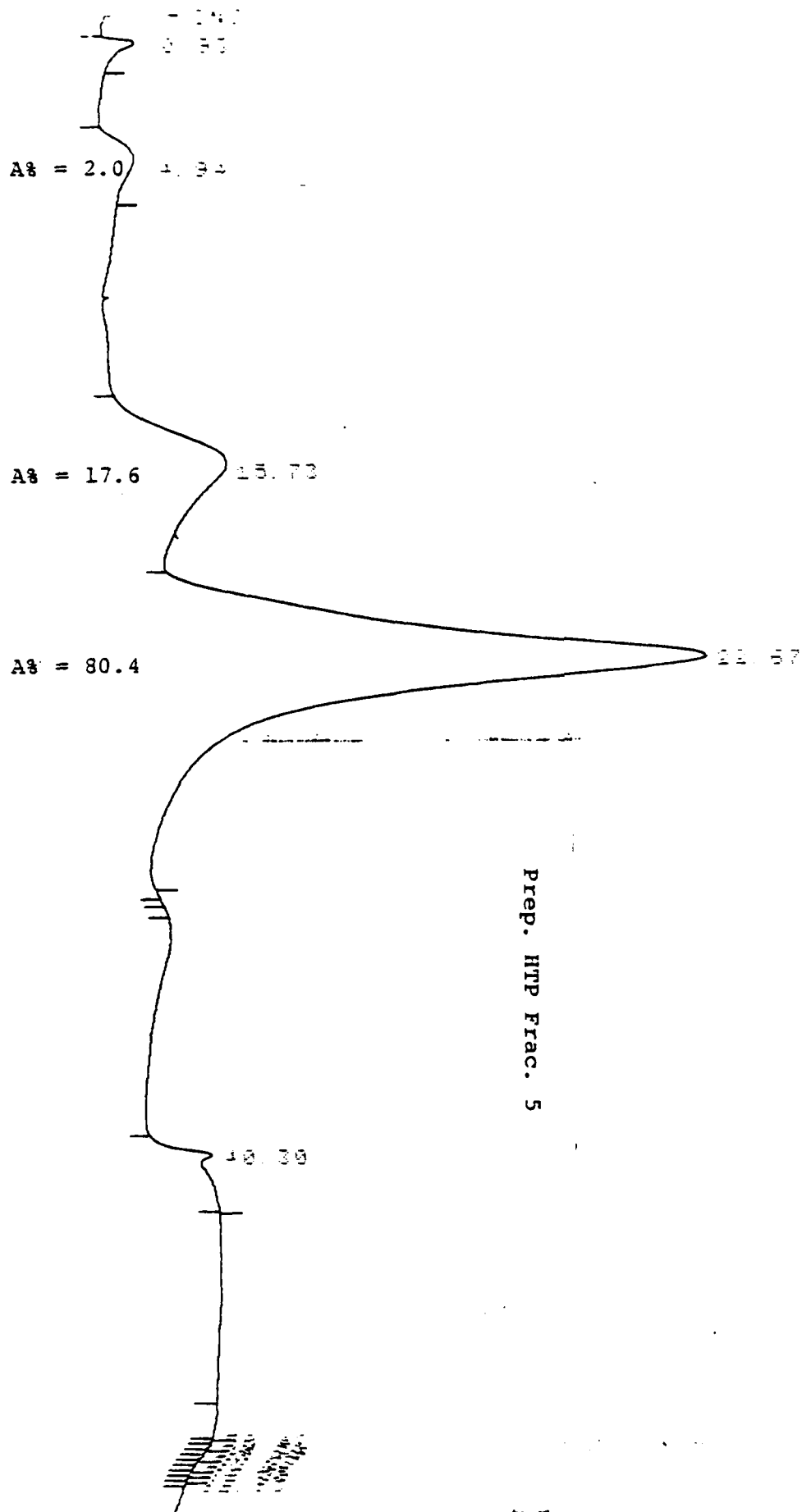
Figure 12

ANALYTICAL HTP CHROMATOGRAPHY OF FRACTIONS SEPARATED BY PREPARATIVE HTP CHROMATOGRAPHY
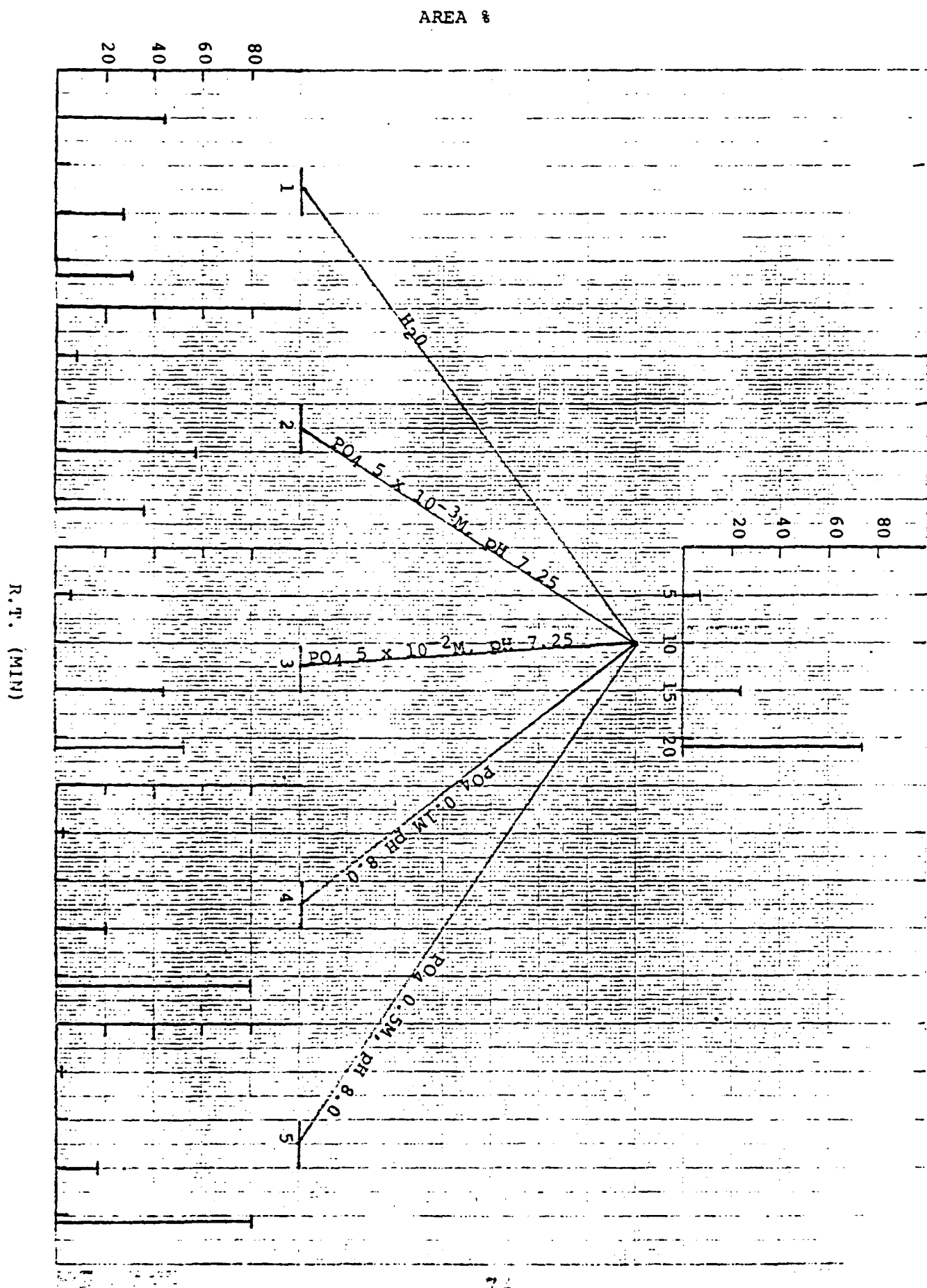
Figure 13
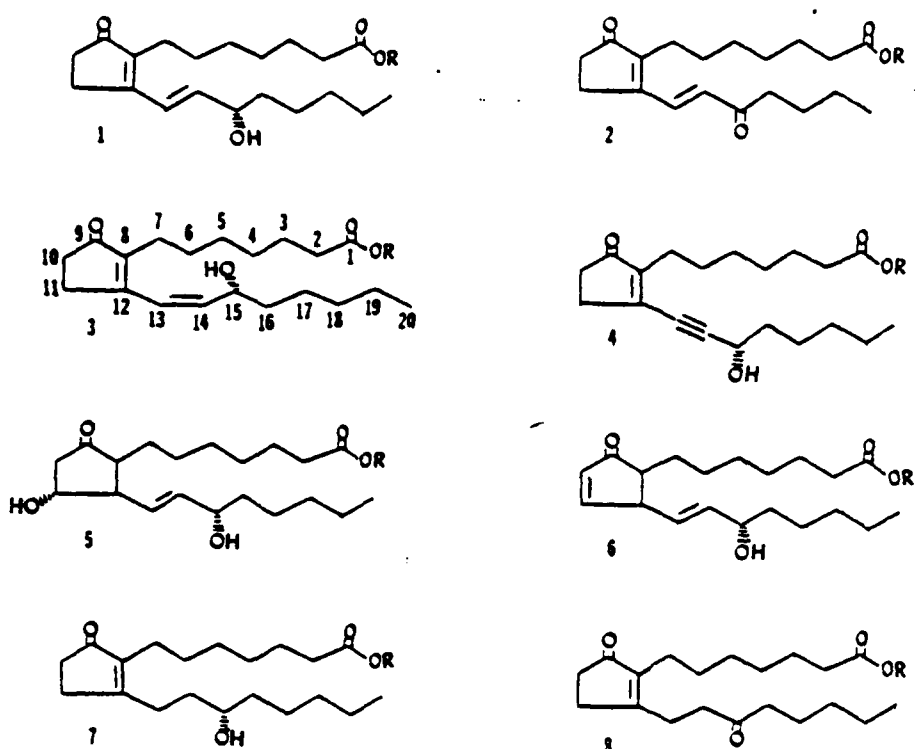
Chemical structures of prostaglandins referred to in text: (*1*) PGB₁, (*2*) 15-dehydro-PGB₁, (*3*) *cis*-PGB₁ (*4*) 13, 14-dehydro-PGB₁, (*5*) PGE₁, (*6*) PGA₁, (*7*) 13, 14-dihydro-PGB₁, and (*8*) 13,14-dihydro-15-dehydro-PGB₁. The conventional numbering system for prostaglandins referred to in the text is illustrated in the example of *cis*-PGB₁ (*3*).

Figure 14 :

Ultraviolet spectra of (A) 15-dehydro-PGB$_1$, (B) PGB$_x$, and (C) 13,14-dihydro-15-dehydro-PGB$_1$. Wavelength of maximum absorption is indicated by number on the spectra. UV absorption of 243 nm for PGB$_x$ is characteristic of a fully substituted cyclopentenone system such as found in C, whereas λ max 296 nm of A indicated the fully conjugated system.
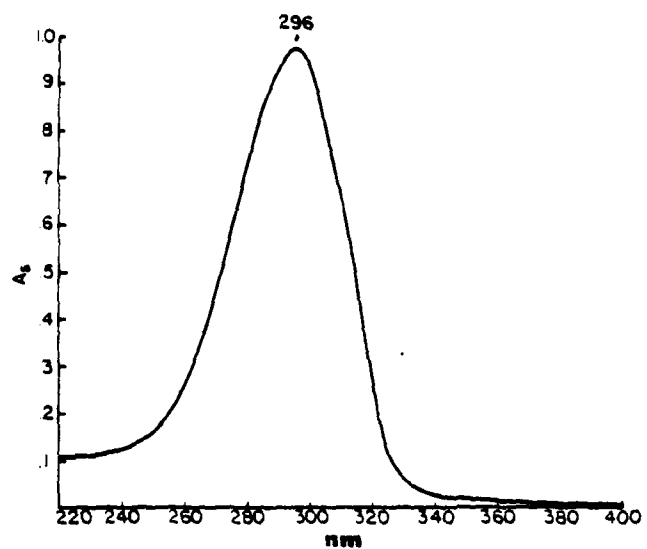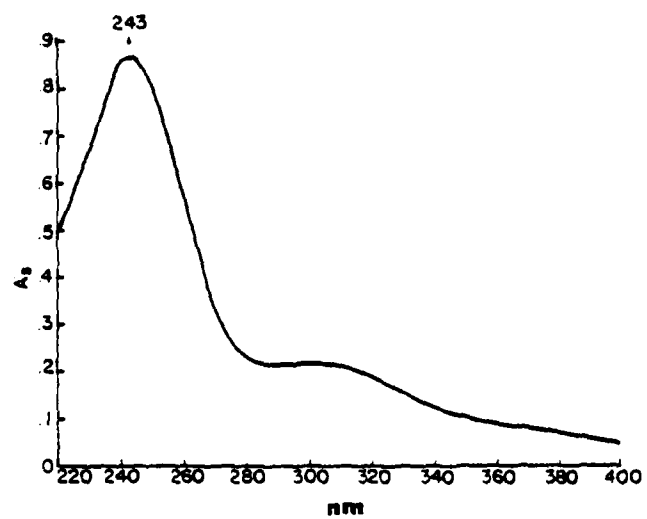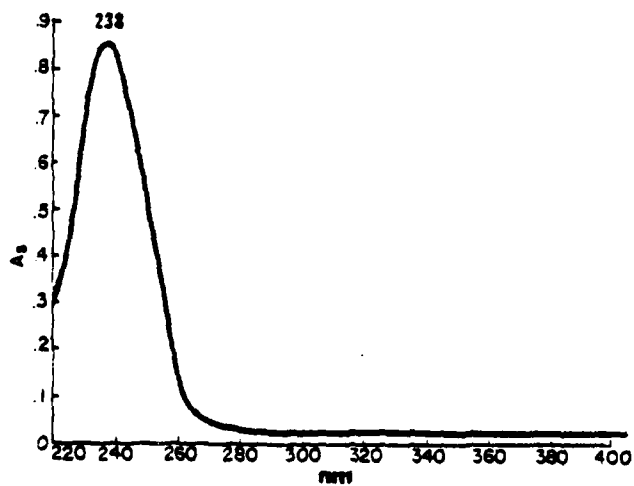
Figure 15 :

Infrared spectra in the carbonyl and conjugated carbon double bond region for (A) 15-dehydro-PGB$_1$, (B) PGB$_X$, (B´) PGB$_X$ methyl ester, and (C) 13,14-dihydro-15-dehydro-PGB$_1$. The absorption at 1630 cm^{-1} in B and B´, and 1640 cm^{-1} in C due to the conjugated C=C stretch, indicates lack of a double bond at the 13,14 position. The absorption at 1585 cm^{-1} in A is characteristic of the fully conjugated system when the 13,14 double bond is present.
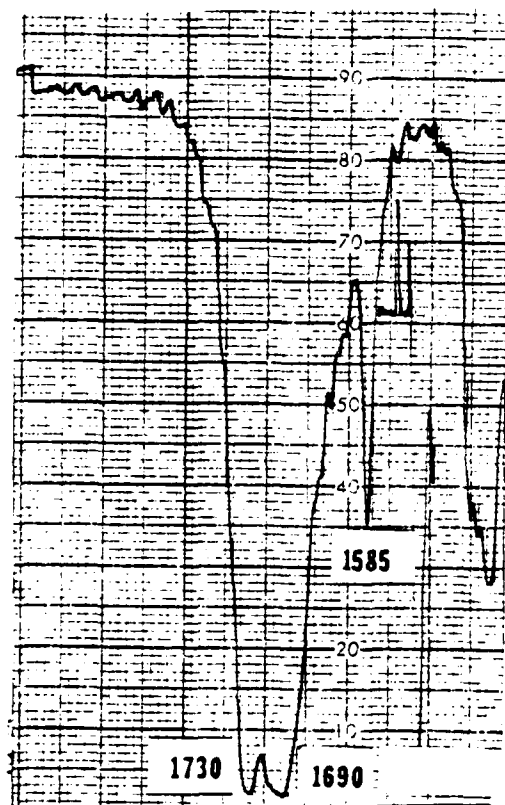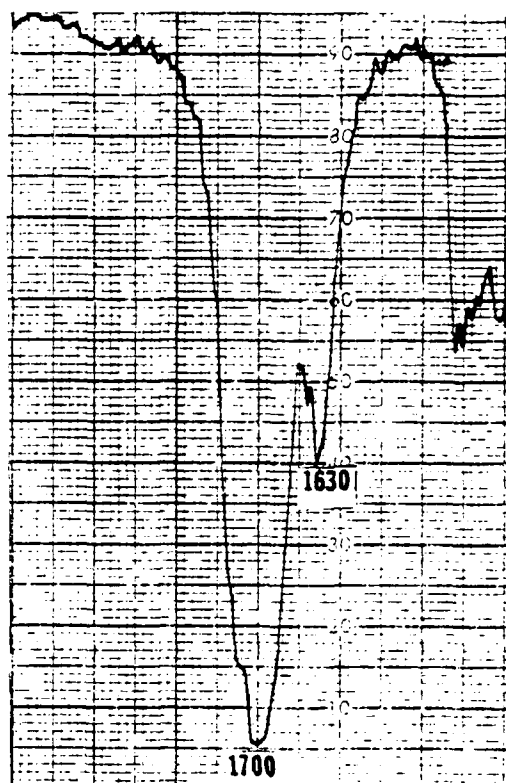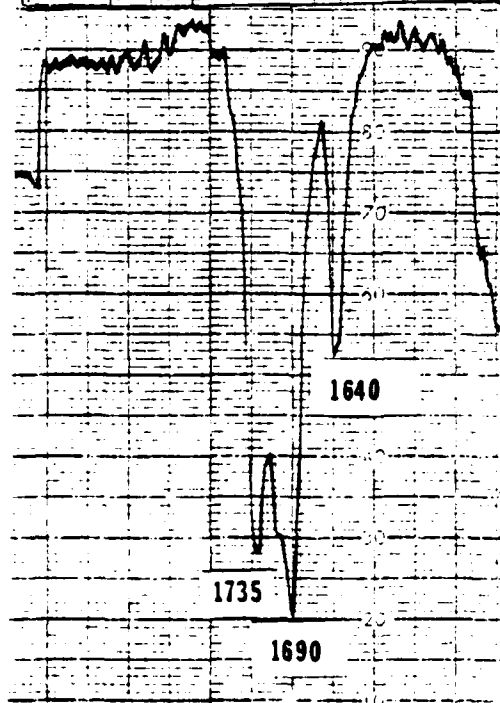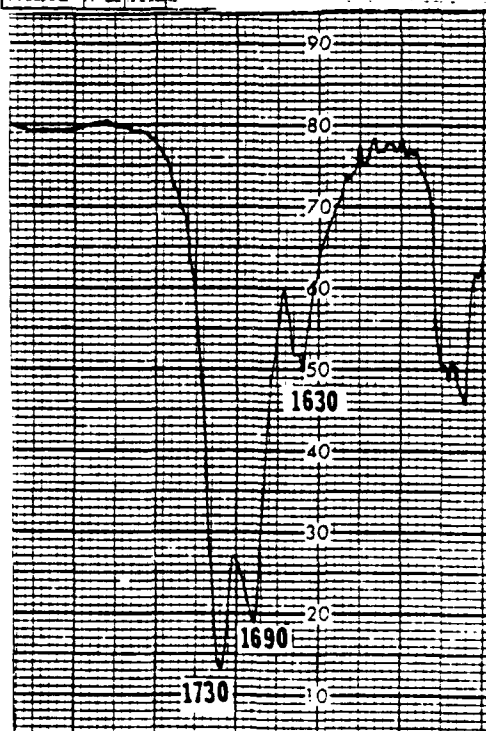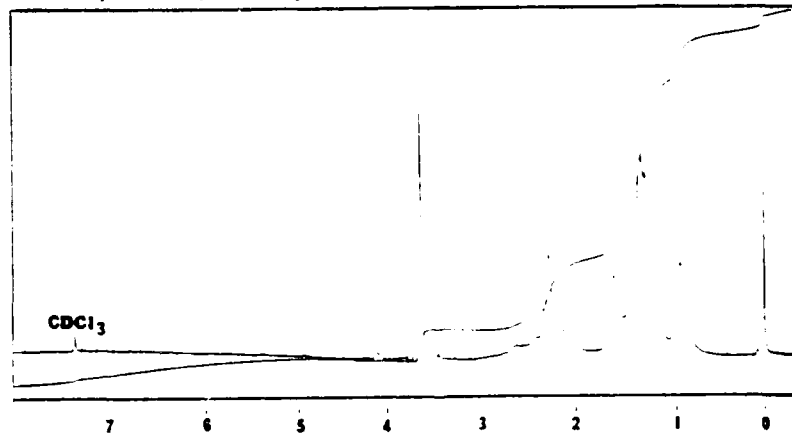
Figure 16 :

360 MHz ^{1}H NMR spectra of (A) 15-dehydro-PGB$_1$, (B) PGB$_X$, (B$'$) PGB$_X$ methyl ester, and (C) 13,14-dihydro-15-dehydro-PGB$_1$. Absorptions for the 13 and 14 vinyl protons of 15-dehydro-PGB$_1$ at 7.65 and 6.59 ppm respectively as observed in A are absent in the spectra of PGB$_X$ and PGB$_X$ methyl ester. The general lack of resolution observed in the spectra of PGB$_X$ and PGB$_X$ methyl ester is compatible with a mixture of closely related oligomers.
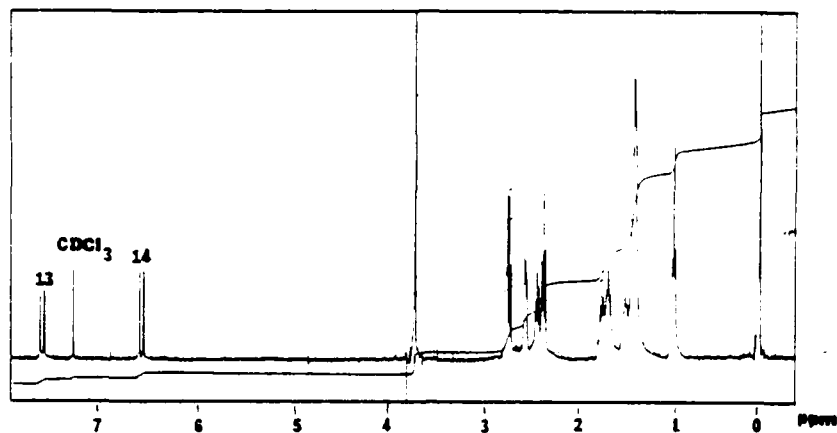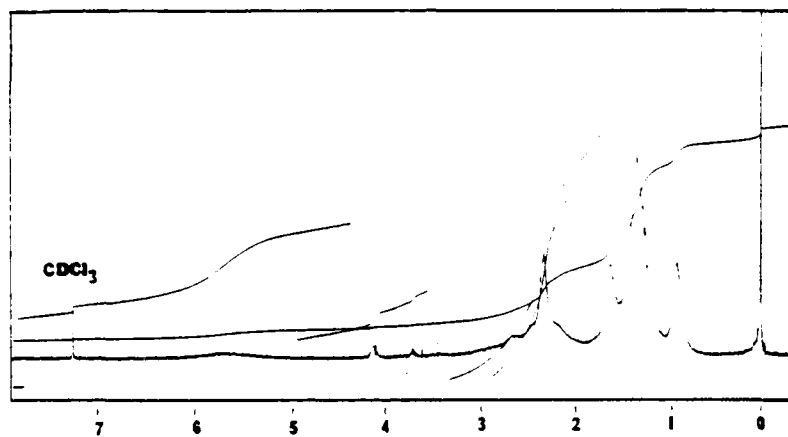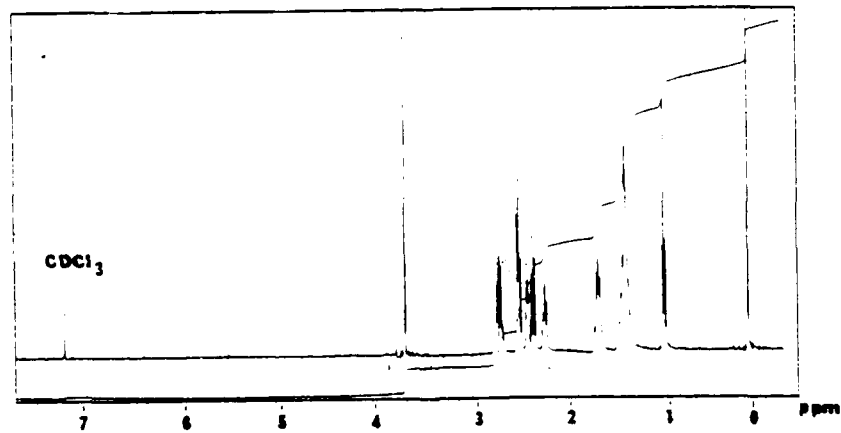
Figure 17 :

20 MHz ^{13}C NMR spectra of (A) 15-dehydro-PGB$_1$, (B) PGB$_x$,
(B´) PGB$_x$ methyl ester, and (C) 13,14-dihydro-15-dehydro-PGB$_1$.
Absorptions at 134.01 and 130.60 ppm for the 13 and 14 vinyl
carbons of 15-dehydro-PGB$_1$ are not present in the spectra of
PGB$_x$ and PGB$_x$ methyl ester and is particularly notable for
those sp^2 carbons associated with the cyclopentenone system.
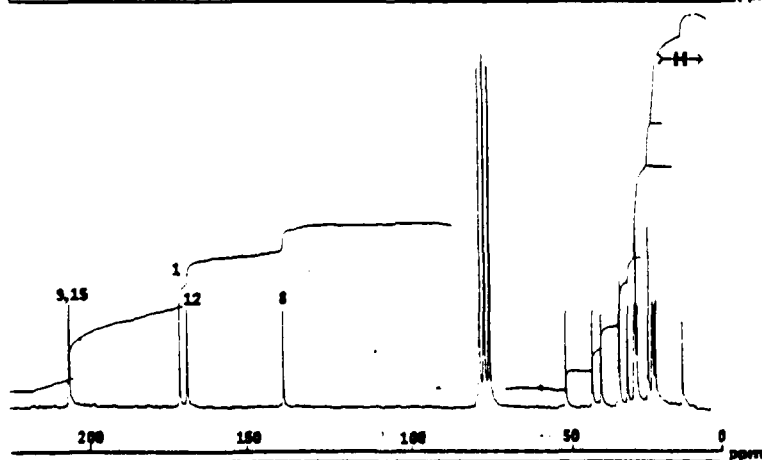Numvers above specific resonances refer to carbon assignments.
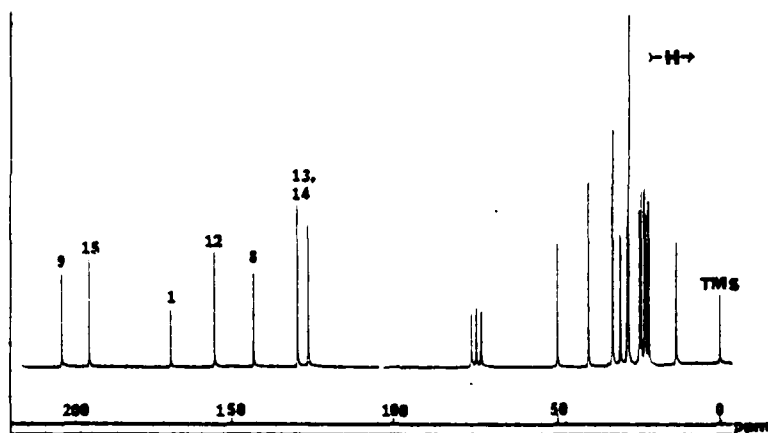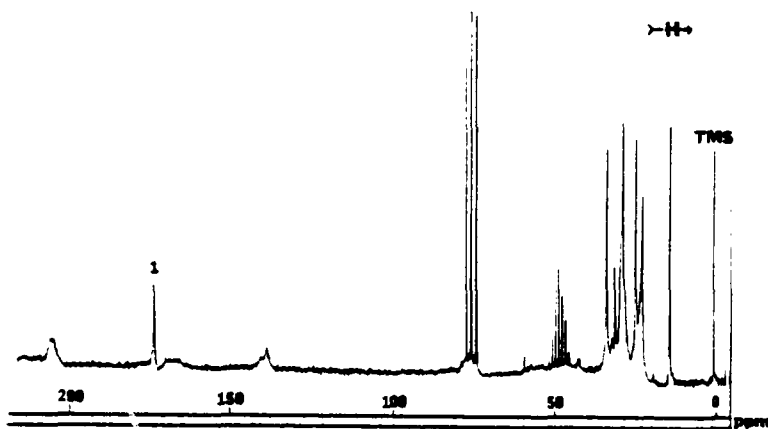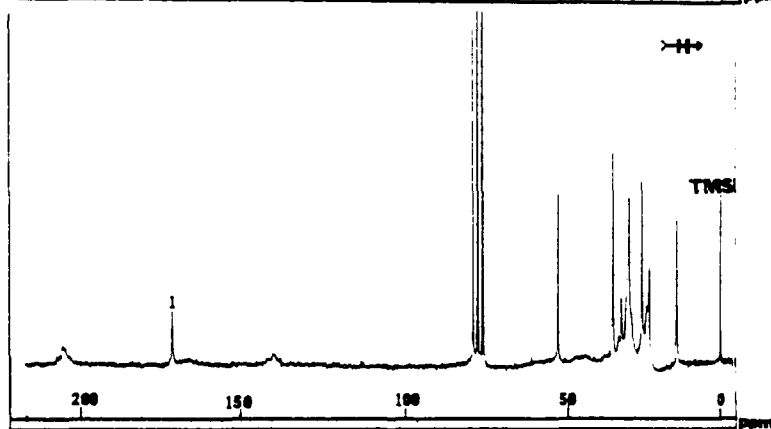
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